

**STUDIES ON THE AETIOLOGY, PATHOGENESIS
AND PREVENTION OF INSULIN-DEPENDENT
DIABETES MELLITUS (IDDM) IN THE
SPONTANEOUSLY DIABETIC BB/EDINBURGH
(BB/E) RAT.**

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ABSTRACT OF THESIS

The BB/E rat spontaneously develops a syndrome closely resembling human Type 1 (insulin-dependent) diabetes mellitus (IDDM). These studies were aimed at determining the sequence of pancreatic events that lead to the mass destruction of pancreatic beta cells, by correlating changes in humoral and cellular immunity with pancreatic morphology in the prediabetic period. In addition, the resulting effects of treatment with the immunosuppressant cyclosporine (CSA) on these events and the incidence of diabetes was examined.

Use of serial pancreatic biopsy within individual rats, and monoclonal antibodies (MoAb) against rat major histocompatibility complex (MHC) antigens, lymphocyte and macrophage subsets has enabled the following sequence of pancreatic events leading to the development of IDDM in the BB/E rat to be described. The first change observed, occurs in some pancreatic lobules about 30 days before the onset of diabetes and consists of increased expression of MHC class I molecules within islets and on the vascular endothelium. Between 2 and 3 weeks before disease onset, expression of MHC class II antigens on vascular endothelial cells accompanies the marked recruitment and accumulation of a population of macrophages at perivascular and periductal sites. These recruited macrophages subsequently infiltrate the adjacent islets thereby initiating insulitis. Further recruitment of other immunocytes including T-helper and T-cytotoxic/suppressor cells, NK cells and B-lymphocytes soon follows such that by 10 days before disease onset, insulitis and resulting beta cell destruction is observed in most of the islets. At this stage, hyperexpression of MHC class I antigens within infiltrated islets is markedly enhanced, extending

beyond the islet periphery into the surrounding exocrine tissue, and although expression of MHC class II molecules was mainly restricted to the infiltrating cells, occasional insulin-containing cells were found to express class II MHC molecules. However, aberrant MHC class II expression on beta cells was never observed in non-infiltrated islets suggesting that it is a secondary rather than a primary initiating event.

This was supported by in vitro studies which showed that islets isolated from 90 day old diabetes-prone (DP) rats expressed MHC class II molecules on 50% of the beta cells when cultured with 100U/ml of rat recombinant interferon-gamma for 4 days. In contrast, expression of class II MHC antigens was never observed on: (i) glucagon or somatostatin cells from any rats; (ii) beta cells from either normal Wistar or diabetes-resistant BB/E rats, or (iii) beta cells from young (30 day old) DP BB/E rats. This suggested the possibility that islets isolated from the older DP rats may have been rendered susceptible to induction of class II MHC antigens by interferon-gamma because of previous cytokine mediated damage in vivo.

Administration of CSA from 30-100 days of age completely prevented the development of diabetes up to 150 days of age and reduced the incidence to approximately 50% of controls (37% v 66%) at 450 days. Whereas islet cell surface antibodies paralleled the development of diabetes, insulin autoantibodies were unrelated to diabetes onset and were unaffected by CSA therapy. Pancreatic biopsies from CSA treated animals showed that CSA had no effect on pancreatic hyperexpression of MHC class I molecules, but inhibited the accumulation of macrophages at extra islet sites, the subsequent recruitment of other immune effector cells and islet infiltration. This resulted in a delay on the onset of

diabetes in some rats and prevention in others. Commencement of CSA therapy at diagnosis of diabetes failed to induce remission of diabetes or reduce the insulin requirements necessary to maintain aglycosuria.

LIST OF ABBREVIATIONS

AChR	Acetylcholine receptor
ADCC	Antibody-dependent cell-mediated cytotoxicity
ALS	Anti-lymphocyte serum
APC	Antigen presenting cells
BB	Bio Breeding
BSA	Bovine serum albumin
Buf	Buffalo rat
CAMC	Complement-dependent-antibody mediated cytotoxicity
CF-ICA	Complement-fixing islet cell antibodies
CIT	Conventional immunosuppressive therapy
CMI	Cell-mediated immunity
ConA	Concanavalin A
CSA	Cyclosporin(e) A
DEX	alpha-1,3,-dextran
DP	Diabetes-prone
DR	Diabetes-resistant
DTH	Delayed type hypersensitivity
EAE	Experimental allergic encephalomyelitis
EAU	Experimental autoimmune uveitis
EBV	Epstein Barr virus
ELISA	Enzyme linked immunosorbent assay
FLU	Fluorescein
GVHD	Graft versus host disease
GVHR	Graft versus host reaction
HBSS	Hanks' balanced salt solution
HEL	Hen egg lysozyme

HGG	Human gamma globulin
HSA	Human serum albumin
IAA	Insulin autoantibodies
ICA	Islet cell cytoplasmic antibodies
ICSA	Islet cell surface antibodies
IDDM	Insulin-dependent diabetes mellitus
IFN	Interferon
Ig	Immunoglobulin
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-2R	Interleukin 2 receptor
Kb	Kilobase ,
LCA	Leucocyte common antigen
LCMV	Lymphocytic choriomeningitis virus
LDV	Lactate dehydrogenase virus
Lew	Lewis rat
LPS	Lipopolysaccharide
LT	Lymphotoxin
MALT	Mucosa-associated lymphoid tissue
MBP	Myelin basic protein
MHC	Major histocompatibility complex
Mls	Minor lymphocyte stimulating
MoAb	Monoclonal antibody
NAD	Nicotinamide adenine dinucleotide
NIDDM	Non-insulin-dependent diabetes mellitus
NK	Natural killer
NOD	Non-obese diabetic
NRS	Normal rabbit serum

PAA	Proinsulin autoantibodies
PBS	Phosphate buffered saline
PGPS	Peptidoglycan polysaccharide
PHA	Phytohaemagglutinin A
PP	Pancreatic polypeptide
RF	Rheumatoid factor
RFLP	Restriction fragment length polymorphism
RIA	Radio-immuno-assay
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
STZ	Streptozotocin
TCR	T cell receptor
T cyt/supp	T cytotoxic/suppressor
T _H	T helper
TNF	Tumour necrosis factor
WBC	White blood cells
WF	Wistar Furth

DECLARATION

I hereby declare that this thesis has been composed entirely by myself. Although part of a research group, the investigations described in this thesis represent the studies in which I have made a major contribution. Acknowledgement of specific assays or procedures not performed by myself is clearly stated throughout the thesis, and therefore unless otherwise stated, the investigations were performed primarily by myself.

Robert Walker

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CHAPTER 1 - GENERAL INTRODUCTION

"Falstaff: Sirrah, you giant, what says the doctor to my water?

Page: He said, Sir, the water itself was a good healthy water; but, for the party that owed it, he might have more diseases than he knew of".

William Shakespeare - Henry IV, Part II
Act I, Scene 2.

HISTORICAL PERSPECTIVES ON DIABETES MELLITUS

Knowledge of diabetes dates back to centuries before Christ. The Egyptian Papyrus Ebers (ca. 1500 B.C.) described an illness associated with the passage of much urine (polyuria). However the name diabetes, meaning a siphon, was given by the Greek Physician Aretaeus of Cappadocia (30 A.D. - 90 A.D.) when he made the first clinical description, describing it as "a wonderful affection, not very frequent in men, being a melting down of the flesh and limbs into urine". Thus, it was long thought that diabetes resulted from kidney disease. The Ayur Veda of Susruta, a Hindu manuscript (6th century A.D.), describes the disease as "madhumeha" or honey urine. However, it was not until centuries later that knowledge of the disease appeared in European writings when Thomas Willis (1621 - 1675) wrote of "the diabetes or pissing evil" and described the urine as "wonderfully sweet as if it were imbued with honey or sugar", thus establishing the name diabetes mellitus. Willis believed that acid salts in the blood, resulting from "immoderate use of wine and cider", were the causative agents responsible for the disease.

In 1776 Matthew Dobson confirmed that the sweetness was indeed due to sugar, but this did not establish the source of the excess sugar. It was Richard Mead (1673 - 1754) who first considered diabetes as a disease of the

liver, an idea subsequently propagated by Claude Bernard (1813 - 78). He observed that the kidney did not produce sugar but excreted the excess into the urine and that it was the liver which normally produced the sugar which it spilled into the circulation. Thus it was thought that diabetes resulted from an overproduction of sugar by the liver. However, another discovery by Bernard; that puncture of the floor of the fourth ventricle of the brain caused glycosuria, was less constructive in its influence since it led to a widely held belief that diabetes resulted from disease of the nervous system.

Within the past century, the association between diabetes and the pancreas has been established. The association began in 1889 when Von Mering and Minkowski reported that pancreatectomy in dogs produced the symptoms of polydipsia and polyuria normally associated with diabetes (1). Prior to this, in 1869, Paul Langerhans, in his doctoral thesis, described discrete groups of cells or "islets" embedded within the main tissue of the rabbit pancreas which could be differentiated from the rest of the tissue by histological means (2). At this time, Langerhans did not record any opinion as to their physiological significance. The name "ilots de Langerhans" was proposed by Laguesse (1893), after he had recognised numerous groups of cells in human pancreas as being similar to those described by Langerhans for rabbit pancreas (3).

Following the observation by Dogiel (1893), that there were no direct connections between these islets and the duct system of the pancreas, Laguesse suggested the possibility that the islets, constituting an endocrine organ, secreted a substance into the "milieu interieur" (3). Further connections, as to the role of the islets of Langerhans in the genesis of diabetes, were established by the observations of Opie (1901) in which

he described the abnormal appearance of the islets from some cases with diabetes (4).

The experiments of Von Mering and Minkowski (1) led to a surge of attempts to prepare an extract of pancreatic tissue that would replace the unknown missing substance of human diabetes. In 1908, Zuelzer prepared a pancreatic extract which he subsequently injected intravenously into 8 diabetic patients which were maintained on a constant diet. The extract was prepared following ethanol extraction of the juice from pressed pancreas which was then evaporated to dryness, with the water soluble residue constituting the final extract for injection (5). Following injection of the extract, the excretion of acetone and sugar in the urine of the patients decreased or disappeared and their general diabetic condition improved. However the injections were followed by severe chills, fever and vomiting, thereby discouraging further experiments.

In spite of these studies, the idea persisted that the pancreatic islets were the source of a hypoglycaemic agent, which prompted Jean de Meyer in 1909 to christen the, as yet, undiscovered substance "insuline" (6), a name adopted by Banting when in 1921 he finally succeeded in isolating the active secretion of the islets.

Frederick Banting met Charles Best in Toronto in the autumn of 1920 and invited him to assist in some experiments aimed at isolating insulin. In May 1921 they commenced work on the project when, in order to eliminate the proteolytic effects of the enzymes derived from the exocrine tissue, they ligated the pancreatic ducts of dogs for 7-10 weeks to allow atrophy of the acinar tissue. The degenerated pancreas was removed, placed in a mortar containing chilled Ringers solution and macerated. The material was then filtered through paper

and the filtrate injected into dogs that had been pancreatectomized a few days earlier. A rapid fall in blood sugar concentration was observed and the general condition of the dogs improved.

To avoid the need for ligation of the pancreatic duct, an attempt was made to prepare insulin from foetal calf pancreas. The pancreas of the calf foetus does not produce trypsin until after the fourth month of embryonic life, and therefore proteolysis of any extracted insulin should be greatly reduced. The insulin solutions obtained were clear and very potent, containing 15-20 units/ml after sterilization.

Although good use of this extract was made experimentally, Banting decided that for clinical trials the source of material should be a commercially viable one. Therefore an acid alcohol extract of normal beef pancreas was prepared and, although less potent than the foetal extract, it proved a suitable source of insulin for use in treating diabetes in man.

The first announcement of Banting and Best's discovery, was made to the Medical Faculty of the University of Toronto on the 11th November, 1921. These findings were sent to the Journal of Laboratory and Clinical Medicine, where they appeared in February 1922 (7). Clinical trials of insulin treatment of human diabetes began in January 1922 under Drs Walter Campbell and Almon Fletcher at Toronto General Hospital, with the results published later in 1922 (8). Large scale production of insulin followed, supervised by Dr Clowes, Research Director of Eli Lilly and Company, working under an exclusive one year licence granted by the Insulin Committee of the University of Toronto. The final steps in the purification of insulin were achieved in 1926 when Abel produced the first crystalline insulin (9) and Scott

(1934) showed that crystallization of the zinc salt was a simple and practical proposition in its commercial manufacture (10).

The discovery of insulin was a major medical triumph, causing death from ketoacidosis to be vastly reduced. Indeed, during the 1920s and for many years afterwards, it was believed that diabetes mellitus had been "cured". However, in the 1940s and particularly the 1950s, diabetic subjects, now in their 4th and 5th decades who had been presumed "cured" by insulin, were developing the so-called specific "diabetic complications" of retinopathy, nephropathy and neuropathy.

After 20 years of diabetes, 80% had retinopathy and of these subjects approximately 50% were destined to die of kidney failure within another 10-15 years. Thus, although insulin provided an effective treatment for sustaining the life of the diabetic patient, the threatening excess morbidity and mortality, with long term insulin therapy, necessitated further research to find a "cure" for the "pissing evil".

Over the past 15 years, there has been an immense renewal of research pertaining to understand the mechanisms underlying the aetiology and pathogenesis of diabetes mellitus. This increased interest in diabetes research reflects and derives its impetus from the tremendous advances that have occurred in basic immunology over the past 30 years. For many years diabetes mellitus per se, being a group of disorders having in common hyperglycaemia, was broadly classified by clinicians into either juvenile-onset (insulin requiring) or maturity-onset (non-insulin requiring).

In the early 1970s evidence was accumulating to indicate that these two forms of hyperglycaemia were, in fact, two distinct disease entities. In particular, the evidence of an HLA association with insulin-dependent (IDDM) but not non-insulin-dependent (NIDDM) diabetes mellitus (11), and the involvement of antipancreatic cell-mediated immunity in IDDM (12), suggested distinctly separate aetiologies for the two conditions.

This thesis will deal virtually exclusively with IDDM or Type 1 diabetes (13) and the increasing awareness of the autoimmune phenomena underlying its aetiology. However, in order to provide a conceptual framework for understanding the aetiology of IDDM, I shall initially review some of the literature pertaining to the history of autoimmunity and its counterpart of immunological tolerance.

AUTOIMMUNITY AND TOLERANCE

Immunological tolerance refers to a specific state of unresponsiveness or hyporesponsiveness which has been induced by prior exposure to a given antigen. When the given antigen is a self antigen then unresponsiveness to self components i.e. self tolerance is established. If self tolerance is somehow terminated, autoimmunity is assumed to ensue with the formation of autoantibodies and T lymphocyte reactivity against self antigens.

HISTORICAL PERSPECTIVES ON AUTOIMMUNITY

The concept of autoimmunity was first postulated in 1901 by Paul Ehrlich and Julius Morgenroth (14). They found that goats immunized with their own erythrocytes failed to produce antibodies against them, and thus formulated the concept of "horror autotoxicus" to explain the animals failure to elicit an immune response against itself. Nevertheless, they recognised that autoimmunity

might occur as an aberration and lead to disease, when they stated: "In the explanation of many disease phenomena, it will in the future be necessary to consider the possible failure of the internal regulation, as well as the action of directly injurious exogenous or endogenous substances" (14).

Subsequently in 1904, Donath and Landsteiner showed that formation of autoantibodies were possible, being responsible for paroxysmal cold haemaglobinuria in syphilis (15). For many years however Ehrlich's dictum that the animal body would not form harmful immunologic reactions against itself was accepted. Half a century later, this concept was expressed by Burnet and Fenner as self tolerance, in which they proposed thymic censorship to prevent the appearance of "forbidden clones" (16, 17).

Early investigations using antigens from diverse sources e.g. spermatozoa for immunization, led to the discovery of cytotoxic antibodies in the animals immunized with the antigens (18), and thereby initiated the concept of autoallergy whereby an animal mounts an immune response against its own self antigens. Early discoveries about eye diseases in which autoallergy was responsible, included phacoanaphylaxis caused by inflammatory reactivity against protein antigens released from a traumatized lens capsule (19).

In 1942 Freund described the efficiency of water-in-oil emulsions for potentiating the antibody response to a variety of antigens (20). It was soon found that incorporation of various normal tissues into Freund's adjuvant could lead to the production of an autoimmune response in the animal. Voisin, reported the development of aspermatogenesis, following the injection of testicular tissue, combined with Freund's adjuvant (21).

Later it was postulated that, with reference to Burnet's clonal selection theory of acquired immunity (17), the use of adjuvants might cause stimulation of so-called "forbidden clones" and that these forbidden clones might augment an immune response against self antigens causing pathological disease. Thus, by the 1950s the conceptual occurrence of autoimmunity and its possible pathological sequelae was gaining increasing credibility as a distinct immunological phenomenon.

In 1957 an attempt to establish criteria for determining the relationship of immunologic phenomena to disease aetiology was presented by Witebsky (22). According to these criteria, an autoimmune response should be considered the cause of a human disease if: (i) the autoimmune response is regularly associated with the disease; (ii) immunization of an experimental animal with antigen from the appropriate tissue causes the animal to make an immune response; (iii) associated with this response, the animal develops pathological changes that are basically similar to those of the human and, (iv) the experimental disease can be transferred to non-immunized animals by serum or by lymphoid cells.

Witebsky's postulates were based on Koch's postulates in bacteriology and reflected the thinking of that period, concerning autoimmunity, when a single cause was believed to be responsible for an autoimmune disease, just as a single microorganism was proven to be the aetiologic agent of an infectious disease.

Recent research now indicates that autoimmune diseases have a multifactorial aetiology, where genetics, environmental and hormonal factors, along with defective immune regulation, combine in concert to produce an autoimmune disease.

DIVERSIFICATION, CO-OPERATION AND REGULATION OF THE IMMUNE SYSTEM

The continued advancement of research into autoimmunity was only possible following the proliferation of basic cellular immunology which, although not exclusively, was in part inspired from Burnet's hypothesis on the "clonal selection theory of acquired immunity" (17). This led to the demise of Ehrlich's template theory of antibody production, with Burnet proposing lymphoid cells being genetically programmed to synthesize one type of antibody. The cell surface antibody would serve as receptor for antigen and proliferate into a clone of cells producing antibody of that specificity. Burnet introduced the "forbidden clone" concept to account for autoimmunity. Cells capable of forming antibody against a normal self-antigen were "forbidden" and presumably eliminated during embryonic development. However, autoimmunity was envisaged as occurring if somatic mutation of lymphocytes generated "forbidden clones" capable of reacting with autoantigens.

There were other notable investigations which greatly influenced our understanding of cellular immunology. In 1948 the role of the plasma cell in antibody production was established by Fagraeus (23). The development of the fluorescent antibody technique by Coons et al, revolutionized the ability to detect antigen within tissues (24).

In 1956 the bursa dependence of antibody formation was demonstrated by Glick et al, following the failure to produce antibodies in animals in which the bursa of Fabricius had been removed (25). This was shortly followed by studies from Miller (26) and Good et al (27) establishing the role of the thymus in the generation of immune responses. Thus the immune system could be separated into a bursa-dependent antibody producing

component and a thymus-dependent cell-mediated component. Later it was found that co-operation between these two limbs was necessary for the production of many immune responses (28, 29). Subsequently the subdivision of the thymus-dependent limb into T lymphocytes, demonstrating helper activity (30) and those described by Gershon and Kondo (31) which brought about suppression, suggested that regulation of the immune system depended in part upon a delicate balance between helper T lymphocytes and T suppressor cells. Thus it was envisaged that disturbances of this balance, creating either hyperactivity of T helper cells or defective suppressor cell function, could result in the production of autoimmunity.

A further restraint directed towards immunoregulation, was proposed by Jerne in his network theory of the immune response (32). This proposes that antibody stimulated by antigen expresses a specific idiotypic that elicits the production of anti-idiotypic antibodies. The idiotypes of these latter antibodies in turn stimulate the production of anti-anti-idiotypic antibodies leading to an open-ended network consisting of a circular configuration of idiotypes and anti-idiotypes. Similar considerations can be made for idiotype regulation of the T cell repertoire where it has been shown that anti-idiotypes can activate the formation of idiotype-specific suppressor lymphocytes (33).

The elucidation of T and B lymphocyte populations and T cell subsets, and their role in immunoregulation, was requisite in providing the platform for further investigations into the aetiology of autoimmunity.

An equally important contribution to the future understanding of autoimmunity, was the demonstrations by Benacerraf and McDevitt (34) and McDevitt and Chinitz

(35) of the significant role played by gene products of the major histocompatibility complex (MHC) in controlling the specificity and regulation of T cell-dependent immune responses.

In 1972 Kindred and Shreffler described experiments indicating that co-operation between T and B lymphocytes was restricted by the MHC (36). In 1973, Rosenthal reported that the presentation of macrophage-associated antigen to lymphocytes, required identity between macrophage and lymphocyte at some portion of the MHC (37).

In 1974, Zinkernagel and Doherty demonstrated genetic restriction for T cell-mediated lysis of virally infected cells, thereby formulating their concept of "dual recognition" for MHC restriction of antigen recognition processes (38). These findings created the central paradox pertaining to T cell tolerance, whereby the immune system must select a repertoire of T cells which can recognize MHC molecules when they are associated with foreign antigens but ignore them when they are not.

SELF TOLERANCE AND INDUCED UNRESPONSIVENESS

The term tolerance was first applied in 1945 by Owen (39), on noting the acquisition of unresponsiveness to allogeneic antigens in dizygotic cattle twins. He observed that binovular twin cattle sharing a common placenta produced a chimaeric state whereby erythrocyte precursors from each twin foetus had become established in the other. This conferred a tolerance towards foreign cells enabling acceptance of skin allografts between the dizygotic twins. Earlier, Traub (40) had noted that mice infected in utero with lymphocytic choriomeningitis virus (LCMV), carried the virus in their blood and tissues throughout life without an apparent immune response,

whereas mice first infected in adult life made a brisk antibody response. These observations led Burnet and Fenner (16) to predict that antigen, introduced into the body during embryonic life, before the immune system had developed, would be mistaken for self, and that it would not evoke antibody formation then or if reencountered later in life. Support for this theory came from the experiments of Billingham et al (41) on "acquired immunological tolerance" to allogeneic skin grafts. Thus, neonatal injections of CBA mouse cells into newborn A strain mice permits the survival of a CBA skin graft in adult life.

In addition, Triplett (42) showed that extirpation of the pituitary from the tree frog during early ontogeny, led to a loss of tolerance to the pituitary such that later grafting of pituitary tissue led to rejection, whereas if only part of the organ was removed the frogs tolerated the graft. These studies led to the widespread belief that self antigens were recognised during ontogeny with resultant establishment of tolerance, and that antigens introduced in the perinatal period preferentially caused tolerance. Tolerance induced in adult animals, by lethal doses of X-irradiation and allo-or xenografting (43), was explained on the postulate that the repopulating lymphoid system recapitulated the events of early ontogeny.

In 1962 the dogma of the uniqueness of the newborn state came under scrutiny. Dresser (44) found that by ultracentrifuging gamma globulin to remove all aggregated material, intravenous injection of small amounts of the deaggregated material into normal adult animals preferentially induced unresponsiveness, whereas the aggregated material favoured an immune response. It was perceived that unprocessed antigen, acting alone, was insufficient to trigger an immune response, but that some accessory stimulus was required. Dresser (44) used the

term "adjuvanticity" for this extra stimulus necessary for activation of immunocompetent cells.

This concept of a stimulus not directly dependent on antigen, was elaborated by Bretscher and Cohn (45) into their two signal theory of lymphocyte activation. This states that lymphocytes require two inductive signals to become activated. One is provided by antigen occupying the receptor for antigen; the other is provided by the action of a helper T cell. If the first signal acts alone, in the absence of the second, the result is a negative signal or tolerance.

Cleveland and Claman (46) were able to induce tolerance to 1-fluoro-2, 4-dinitrobenzene-modified self antigens in mice by injecting haptenised cells intravenously. Surprisingly, an additional injection of concanavalin A (Con A), a lectin capable of inducing interleukin-2 (IL-2) production, changed the tolerogenic nature of the haptenised cells into an immunogenic one. This observation was followed up by Malkovsky et al (47) who showed that administration of exogenous IL-2 to newborn CBA mice, annulled the tolerogenic potential of semiallogeneic cells previously injected into the CBA mice. Thus, since newborn mice fail to produce IL-2 (48) and the induction of neonatal tolerance could be abrogated by exogenous IL-2, neonatal tolerance was interpreted as a consequence of the exposure of the immune system to antigen in the absence of IL-2.

Zonal Tolerance and Suppression

As mentioned earlier, the elucidation of the immune system into T and B lymphocyte compartments revolutionised the study of immunology. The impact of these discoveries on research into tolerance was equally profound. Weigle et al (49) showed that human gamma

globulin, injected into adult mice, produced tolerance in the T cell compartment more rapidly and at a much lower dose of antigen than for B lymphocytes. The greater ease of tolerance induction in T cells was similarly reflected in the system of high-zone and low-zone tolerance established by Mitchison (50).

In 1949, Felton (51) had reported that suprainmunogenic amounts of pneumococcal polysaccharide could cause non reactivity in normal adult mice. However, at that time the concept of the uniqueness of the perinatal state persisted and this observation was overlooked.

In contrast to Felton's studies, the equally unexplained observations of Shellam and Nossal (52), where femtogram amounts of flagellar antigens were able to induce tolerance, were subsequently explained by the presence of T suppressor cells (31).

During the last 15-20 years, numerous models of unresponsiveness involving suppressor cells have been described (for reviews see references 53-55). Eardley et al (56) demonstrated, in an in vitro model, that exposure of T cells to high doses of sheep erythrocytes led to the stimulation of a regulatory subset which was able to activate other T cells into becoming functional suppressor cells. The target antigen for the modulation of the humoral response appeared to be the helper T cell.

Jandinski et al (57) showed that class I histocompatibility antigens were required on the carrier cell to elicit active suppressor cells, and Pierres et al (58) demonstrated that H-2 negative cell lines were inefficient at suppressor cell induction. Thus the crucial role of MHC antigens in regulating immune responses, mentioned previously, was equally apparent for restricting the transfer of suppression.

Parameters of Induced Unresponsiveness

Although the induction of tolerance in the newborn is no longer unique, nevertheless, tolerance is more readily induced and of longer duration in immature animals than in adult animals. This probably reflects the differential sensitivity of immature and mature lymphocytes to tolerizing signals transduced via their membrane antigen receptors. The studies of Dresser (44) inferred the importance of antigen composition, whereby materials which are readily phagocytosed are potent immunogens, whereas those which are poorly phagocytosed are highly tolerogenic.

Further, Pike et al (59) showed the effects of altering the molecular arrangement of haptens and carrier molecules on their efficacy as tolerogens. Thus, by coupling increasing numbers of fluorescein (FLU) residues as haptens onto either human gamma globulin (HGG), the $F(ab^1)_2$ fragment of HGG or bovine serum albumin (BSA), they found the higher the hapten density, the lower the concentration of antigen required for induction of tolerance.

The early investigations of Sulzberger (60) and Chase (61), indicated an important consideration in inducing tolerance as being the route in which the antigen was administered. Antigens administered systemically, by intravenous or oral routes, tend to favour unresponsiveness, whereas local deposition of antigen intradermally or subcutaneously is usually immunogenic. Chiller et al (62) demonstrated that maintenance of specific unresponsiveness to an antigen depended on the continual presence of the antigen, with tolerance diminishing as the antigen concentration fell below a critical level.

Finally, Mitchison (50) demonstrated that for some T dependent antigens, tolerance could be induced at two dose ranges; one lower and one higher than that used for optimal immunization. As such, the dose, composition, route of administration and persistence of antigen, along with the age of the animal, are all important parameters to be considered in models for studying the mechanisms of tolerance induction.

MECHANISMS OF TOLERANCE

Many models have been developed to study the mechanisms underlying the induction of tolerance.

I do not intend to review this vast area but suggest, to the interested reader, the reviews of Weigle (63), Nossal (64) and Scott (65). Use of these models has led to the provision of several possible mechanisms for tolerance. The three most commonly pursued being: (i) clonal anergy, reflecting functional inactivation of self reactive lymphocytes; (ii) clonal suppression, mediated by antigen specific T suppressor cells or regulatory elements of an idiotypic network and (iii) clonal deletion where self reactive lymphocytes are assumedly eliminated from the pre-immune repertoire. It must be stressed that none of these mechanisms should be thought of as mutually exclusive, nor universally applicable, for all antigens. Indeed, given the importance of avoiding uncontrolled autoimmunity, it would not be surprising that the immune system has evolved whereby provision of a suppressor system may act as a fail-safe mechanism to an initial repertoire-purging mechanism. Presently, controversy in distinguishing between these mechanisms still persists.

One of the difficulties contributing to this controversy stems from the difficulty in determining the fate of anti-self lymphocytes which are generated at very low

frequencies. Thus, although self reactive lymphocytes can be detected in secondary lymphoid tissues (66), their functional significance remains unclear for two reasons. First, their detection has relied on the use of complicated functional assays, where measurement of the reactivity (or lack of it) cannot distinguish between clonal deletion, anergy or suppression; secondly, the cells represent an unknown fraction of the total output of anti-self lymphocytes from the thymus or bone marrow.

Several recent papers have circumvented these difficulties and illustrate how different mechanisms may be applicable in different systems and, indeed, suggest that the mechanisms of tolerance are different for T cells and B cells.

Anergy in the B Cell Component

Goodnow and colleagues (67) were able to generate high frequencies of anti-self lymphocytes in the pre-immune repertoire, by introducing immunoglobulin genes into the germline of transgenic mice. Transgenic mice carrying rearranged immunoglobulin heavy and light chain genes, encoding a high-affinity anti-lysozyme antibody, were created. This resulted in having mice in which most of the B lymphocytes bind with high affinity to hen egg lysozyme (HEL).

A second transgenic animal was established in which the gene encoding lysozyme was introduced into the germ line, yielding mice for which lysozyme was a tolerated self component in both the T and B cell compartments. By mating the two kinds of transgenic mice, "double-transgenic" offspring carrying lysozyme and immunoglobulin transgenes were produced. Many lysozyme specific B cells were found in the spleen and lymph nodes of these mice. However, despite the many antigen

specific B cells, the mice failed to make antibody responses to lysozyme.

To test the functional capability of the B cells, small numbers of spleen cells were transferred into non-transgenic mice previously exposed to HEL and therefore containing lysozyme specific helper T cells. In this environment, the lysozyme specific B cells still failed to make an antibody response. The authors argue that the inability to respond is a property of the B cells and not of another transferred cell such as a suppressor T cell, because only 10^5 cells were transferred. Thus, in this example, tolerance was achieved by rendering anergy of the B cells.

Deletion in the T Cell Component

Another approach to follow the fate of self reactive lymphocytes, has involved the identification of naturally occurring situations in which the frequency of T cells, specific for certain polymorphic cellular antigens, is relatively high and can be measured directly by anti-T cell receptor (TCR) monoclonal antibodies (MoAb)(69-72). The MoAb KJ23a reacts with TCR's utilizing the $V\beta$ segment $V\beta 17a$, and T cells bearing $V\beta 17a^+$ receptors react with very high frequency with the MHC class II protein, IE (68).

Kappler et al (69) have used this MoAb to follow the fate of T cells expressing $V\beta 17a^+$ receptors in mice which have a functional IE molecule. Their results indicated that cells with $V\beta 17a^+$ receptors occur at normal levels among immature thymocytes, but are severely depleted among mature thymocytes and peripheral T cells. Therefore, clonal deletion as a mechanism for inducing T cell tolerance to self MHC antigens was indicated.

Subsequently, Kappler et al (70) and MacDonald et al (71) reported a similar mechanism for tolerance induction to the Mls (minor lymphocyte stimulating) antigen. T lymphocytes reactive with the product of the Mls^a allele use a predominant TCR beta chain variable gene segment, V β 8.1 (70) or V β 6 (71). It was found that mice expressing the Mls^a allele had few or no T cells bearing receptors containing the specific β chain variable regions V β 8.1 or V β 6. Thus tolerance in this system was mediated via the intrathymic elimination of self reactive cells rather than by clonal anergy or peripheral suppression.

A further paper using the anti-V β 6 MoAb on cryostat sections of thymus, indicated that V β 6 expressing cortical T cells were present at high density in both Mls^a and Mls^b mice, but did not appear in the medullary region of Mls^a animals (72). Tolerance induction therefore appeared to occur at the cortico-medullary junction, where it may be mediated by dendritic cells which are found in this region.

Selection and Tolerance - The Paradox

The above mentioned papers (69-72) also shed some light in explaining the paradox of MHC restriction and tolerance mentioned previously. Thus during selection in the thymus, cells are both positively selected and deleted because of interactions between their receptors and what appears to be the same ligands, the products of self MHC.

Traditionally, the paradox has been explained by suggesting that positive selection picks out thymocytes bearing receptors with a wide range of affinities for self MHC. Tolerance on the other hand, deletes only those thymocytes bearing receptors with high affinity for self MHC, allowing cells with low affinity receptors to

escape into the periphery where they form the pool of self restricted mature T cells.

Marrack and Kappler (73) have suggested an "altered ligand" hypothesis to account for the paradox of selection for, and tolerance to, self MHC. The hypothesis uses the recent discovery by Bjorkman and colleagues (74) that MHC proteins bind peptides in a pocket on the external surface of the molecule, and the concept that in the absence of foreign antigen, self MHC is bound to peptides derived from self proteins (75). In addition, the spectrum of peptides bound to MHC may vary from tissue to tissue (76). Thus, self MHC molecules on thymic epithelial cells are not bound to the same spectrum of self peptides as MHC molecules on B cells and macrophages. Therefore thymocytes may be positively selected by interaction of their receptors with self MHC plus epithelial cell peptides, but clonally deleted by interaction of their receptors with self MHC plus peptides expressed on bone marrow derived cells such as dendritic cells at the cortico-medullary junction.

Evidence Against Clonal Deletion

Clonal deletion however cannot be true for all models of unresponsiveness. Transplantation models have involved the induction of tolerance to transplanted allogeneic tissue, without the loss of T cells reactive to the foreign MHC antigens(77). In addition, numerous examples document the presence of lymphocytes from normal individuals and animals which are capable of binding to self antigens (78, 79). Autoantibodies are found in the sera of normal individuals (80), and in mice autoantibodies that react with multiple organs are a common feature of the normal immune repertoire (81). Moreover, autoantibody production by lymphocytes from

normal individuals (82) and animals (83) can follow stimulation by non specific mitogens.

The affinity of antibodies varies greatly and cross-reactivities among unrelated antigens and antibodies are common. Also, with the number and diversity of self antigens being very large, clonal deletion of all cells with any self reactivity could, via cross reactivity, result in eliminating the total immune repertoire (64). Thus consideration of clonal deletion as the mechanism of tolerance, should be qualified with exemptions such as for sequestered antigens or antigens present in low concentrations in extracellular fluids. Therefore, when considering a selective immune system, geared to react to the unknown where factors determining the generation of immunologic diversity never cease, there should be a mechanism in adult as well as foetal life for clonal deletion. Thus, although the controversy between the mechanisms of self tolerance persists, it would not be surprising to find that the immune system has developed a cascade of mechanisms to operate at various stages of ontogeny. These mechanisms need not be seen to act in isolation of one another, but rather, may act in concert to prevent the "horror autotoxicus".

AUTOIMMUNITY : PATHOLOGIC OR PHYSIOLOGIC

The theories, hypotheses and suggestions propagated to account for the development of autoimmune diseases, are as abundant as the diseases themselves. Indeed, this is perhaps as it should be since the multifactorial nature of autoimmune diseases allows different mechanisms to apply to different diseases, and even can permit a heterogeneous pathogenesis between individuals with the same diseases. However, there must be distinctions drawn between autoimmunity per se, and the establishment of autoimmune disease, for not all autoimmune phenomena are

necessarily pathologic. The self recognition that takes place in the normal immune response represents a fundamental form of autoimmunity. For cells to communicate with one another in immunoregulation, they must recognise and interact with self determinants on each others surface. Likewise, anti-idiotypic antibodies, against antibody formed in response to selected antigens, represents another form of physiologic autoimmunity (32).

Grabar (84) has suggested that autoimmunity represents a normal mechanism for the transport of autoantigen not degraded by the body's enzyme system. It is postulated that immune phenomena developed in evolution as physiologic rather than as defence mechanisms, to handle metabolic and catabolic substances. In his view, both autologous and exogenous materials of the body undergo degradation by specific enzymes, such as autolytic enzymes, under normal conditions. He suggests that those substances not degraded in this manner elicit the formation of antibodies that serve as transporters and assist in the preparation of such substances for phagocytosis.

The removal of senescent red blood cells by macrophages, after coating the cells with IgG, may represent a physiologic role for the immune system (85). In addition, the low levels of autoantibodies detectable in the sera of normal individuals (80), may reflect breakdown or alteration of tissue components by external factors. Their purpose could be to merely facilitate the removal of the altered products.

A further distinction is required to separate the production of autoantibodies per se, from the pathogenicity of the autoantibody formed. Anti-heart antibodies may be generated after the heart has been

injured by a pathologic process such as myocardial infarction (86). As such they represent mere sequelae of a pathologic event.

Autoreactive plaque forming cells (PFC), specific for DNA, can be detected simply by culturing murine spleen cells with or without the presence of mitogen. When the specificity of the PFC is ascertained by plaque inhibition, it is found that they are of low affinity (87). However, similarly detected PFC found in spleens of autoimmune susceptible mouse strains are of high affinity. Thus autoantibody per se need not necessarily be deemed the avoidable "horror" initially predicted by Ehrlich. High affinity autoantibody which can be pathologic, may only be found when the immune system suffers a major disturbance.

Pathogenic sequelae may be generated against self antigens either as (i) an abnormal immune response to a normal self antigen, (ii) a normal immune response to antigens which show cross-reactivity with antigenic determinants on normal cells, or (iii) following exposure of sequestered antigens. Sequestered self antigens, such as those of the testes, brain and lens of the eye, are not normally in contact with circulating immunocompetent lymphocytes. However, traumatic injury or infection may expose antigens of these tissues to immunocompetent cells, leading to activation of an autoimmune response against these normal tissue antigens. Thus, phacoanaphylaxis caused by inflammatory reactivity against protein antigens released from a traumatized lens capsule (19), and aspermatogenesis induced by the formation of antisperm antibodies following vasectomy (88), may occur.

In an attempt to account for the diverse concepts of autoimmunity, such as those of Ehrlich and Morgenroth (14) and Burnet (17), that stressed the pathogenic role of autoimmunity, as well as the theories of Boyden (89) and Grabar (84) that emphasized physiologic autoimmunity, Beutner et al (90) developed their "unifying concept of autoimmunity". They state that common features of physiologic and pathologic autoimmunity include (i) the demonstration of autoantibodies or cell-mediated immune reactivity by in vitro assays using the hosts self tissue antigens, (ii) antigen identification, and (iii) evidence of in vivo reactivity between homologous antigen and the host autoimmune response.

The key factor in distinguishing between physiologic and pathologic autoimmunity is whether the autoimmune reactivity serves a helpful function or constitutes part of a pathologic process. They describe autoantigens as either accessible (A-self) or hidden (H-self). Then reflecting on Witebsky's postulates (22), Beutner et al suggest that pathologic and physiologic autoimmunity differ in whether or not (i) the antigen is accessible or hidden, (ii) the autoimmune response is specific for a certain disease, (iii) pathologic changes by anti-accessible responses can be induced passively in experimental animals, (iv) anti-hidden self antigen responses can actively prevent a host from the injurious action of excessive quantities of undegraded hidden self antigens, and (v) autoimmune responses and the associated diseases can be induced in normal subjects by actively immunizing them with the autoantigens.

Subsequently, Smith and Steinberg (91) described a further conceptual framework for considering autoimmune diseases. They emphasized that controlled self reactivity represents a normal event; that self reactions are important mechanisms of normal immune reactivity and

normal immune regulation; and that disease results from quantitative abnormalities (e.g. amount of stem cell proliferation; degree of autoantibody production or levels of immune complexes). They also stressed that autoimmune diseases have a multifactorial aetiology rather than a single cause. Individual genes may predispose an individual to develop certain abnormalities associated with an autoimmune disease, but environmental factors that stimulate the immune system and interfere with normal immune regulation, are among the precipitating factors that lead to the manifestation of disease.

MECHANISMS OF AUTOIMMUNITY

As mentioned previously, tolerance to normally sequestered antigens can be broken following any trauma which allows contact of those antigens with the immune system. Further mechanisms which can induce autoimmunity include any procedure which can bypass the requirement for specific T cell help when immunocompetent B cells are present.

Cross-Reacting Antigens

Extensive research from Weigle and his colleagues has demonstrated that T cell tolerance can be overcome by, for example, the use of cross-reacting antigens (63, 92, 93). Thus, rabbits made unresponsive to BSA at birth, make an antibody response to BSA when injected as adults with human serum albumin (HSA)(92). Since HSA shares only 15% homology with BSA, it leaves antigenic determinants free to permit T helper cells to provide support not only for B cells reacting specifically with HSA, but also for those B cells reactive against cross-reactive determinants on BSA. In addition,

unresponsiveness to BSA can also be circumvented using BSA complexed with anti-HSA antibodies (93). Equally, autoantibodies against self thyroglobulin may be elicited in rabbits following immunization with cross-reacting antigens on bovine thyroglobulin (94). Infection with group A beta-haemolytic streptococci can lead to an immune response in susceptible individuals, where antibodies against the streptococci can cross-react with antigens of the heart (95).

Viruses, Drugs and Polyclonal Activation

The combination of autoantigens and antigenic determinants from exogenous substances, may form immunogenic units that are capable of stimulating T helper cells that can activate B cells to produce autoantibody. Types of helper antigenic determinants include agents such as bacteria, viruses or drugs. Autoimmunity may occur following interaction of viruses with the immune system. Epstein-Barr virus (EBV) infects B lymphocytes causing B lymphocyte proliferation and inducing immunoglobulin secretion. This leads to induction of autoantibodies which tend to react with more than one organ or tissue (96). Shared determinants between reovirus Type 1 and certain tissue antigens results in the formation of various autoantibodies (97). Polyclonal activation is also observed in mice infected with lactate dehydrogenase virus (LDV)(98). Cross-reactive autoimmune responses against intermediate filaments are found following infections with vaccinia (99), or measles and herpes simplex viruses (100).

Antibodies formed to paramyxovirus also react with antigens found on brain tissue (101). Myelin basic protein (MBP) is an immunogen of importance in the genesis of demyelinating diseases, where the encephalitogenic site is a known decapeptide. When viral

proteins that have been sequenced were screened by computer for sharing of 6-10 consecutive amino acids with the encephalitogenic site of MBP, shared sequences were found in polypeptides of many common viruses, including adenoviruses, influenza A, EBV and measles virus (102). Presumably, however, depending on additional factors, only a few of these shared sequences would be able to induce significant autoimmune responses, otherwise autoimmune disease would be an all too common occurrence following viral infections.

Certain drugs may elicit the production of autoantibodies in otherwise normal individuals. The drug or its metabolites may bind to an autoantigen, the combination of which may activate T helper cells to provide help for B cells specific for the autoantigen to make autoantibodies. For example, hydralazine or procainamide-treated patients can form antinuclear antibodies that resemble those of patients with systemic lupus erythematosus (SLE)(103). Increased helper cell activity has been reported in procainamide-induced lupus (104). This response may be brought about by the drug or its metabolites interrupting the normal immunoregulatory balance between T helper and T suppressor cells. Thus procainamide may inhibit cyclic AMP, an effect which can either stimulate helper cell activity, or equally, inhibit T suppressor cells (105). Further, alpha-methyldopa is a drug which can cause autoimmune haemolytic anaemia (106). The drug activates cyclic AMP, an effect which inhibits suppressor cells. Diminished suppressor activity can allow helper T cells to activate immunocompetent B cells to produce antibodies which often have an antirhesus specificity, to combine with the drug attached to the cell surface; fix complement and thereby induce a bystander type of complement-mediated lysis (107). Thus, autoimmunity may result from either stimulatory or inhibitory effects of a

drug on immunoregulatory cells. In addition, anti-lipomodulin autoantibodies found in patients with SLE, rheumatoid arthritis and dermatomyositis (108), can disrupt immunoregulation by reducing the formation of prostaglandins, and hence impair prostaglandin-dependent suppressor cells (109).

Bacterial endotoxins; lipopolysaccharides (LPS) are substances whose biological effects on immunological processes permit a response to a variety of antigens, in the absence of specific T helper cells. Endotoxins may exert mitogenic effects on B lymphocytes (110). They can substitute for helper T cell function in the antibody response to sheep erythrocytes (111), or bypass the requirement for T cells in a hapten-carrier system (112). Also, induced unresponsiveness to human gamma globulin can be terminated by the administration of the antigen with endotoxin (113). Likewise, any other vehicle which stimulates lymphocytes non specifically, such as immunologic adjuvants or allogeneic cells that mediate graft-versus-host reactions (GVHR), may activate the formation of autoantibodies, through bypassing T cell specificity.

If parental strain T lymphocytes are injected into adult FI hybrid mice, the recipients are genetically tolerant of parental donor strain cells. However, the grafted T lymphocytes can react against allogeneic MHC antigens of the host and induce a spectrum of pathological changes, resulting in graft-versus-host disease. In this situation, it is found that a spectrum of autoantibodies, similar to that found in SLE i.e. antibodies against epithelial cells, erythrocytes, thymocytes, nuclear antigens and double-stranded DNA, are produced (114).

Autoantigen

A further constraint in the generation of autoimmunity is the nature and role played by the autoantigen (115). Thus it was found that a mouse autoreactive T cell clone, specific for murine thyroglobulin, differed in its ability to recognize human thyroglobulins depending on their degree of iodination. Thyroglobulins with low levels of iodine reacted poorly with the T cells (116). It was also shown that poorly iodinated thyroglobulin is unable to induce thyroiditis in mice when injected with adjuvant, although it is only slightly less effective than iodinated thyroglobulin in eliciting autoantibody production (116). Thus, iodination of thyroglobulin may be necessary for recognition by T cells involved in the pathogenesis of thyroiditis, but not for all T helper cells involved in autoantibody production.

Equally, autosensitization of serum IgG may be important in the pathogenesis of rheumatoid arthritis. Recently, Parekh et al (117) reported reduced glycosylation of oligosaccharides in the IgG of patients with both juvenile and adult onset rheumatoid arthritis. The defect in glycosylation is associated with the oligosaccharides of the Fc portion rather than the Fab fragment. The two N-linked Fc oligosaccharides, one originating from each C γ 2 domain, form a bridge to hold the C γ 2 domains apart, while the terminal galactose residues sit in a pocket on the surface of each domain (118). This glycosylation defect was not a general feature of rheumatological disorders, being confined to rheumatoid arthritis, tuberculosis and Crohn's disease. The low galactose levels were not due to chronic excessive IgG synthesis, since normal glycosylation is seen in primary SLE and leprosy diseases where raised IgG levels exceed those seen in rheumatoid arthritis (117).

Axford et al (119), reported that the activity of galactosyltransferase, the enzyme which catalyses the addition of galactose to the oligosaccharide chains, was considerably lower in the circulating B cells of patients with rheumatoid arthritis. Diminished galactosyltransferase activity is also found in the peripheral blood cells of patients with untreated tuberculosis and active Crohn's disease (118). These studies suggest a possible mechanism for the pathogenesis of rheumatoid arthritis, where IgG rheumatoid factors form self-associated complexes which stimulate a chronic inflammatory response resulting in the formation of an erosive pannus. Thus, since the Fab oligosaccharides of rheumatoid patients are glycosylated normally, self-associated complexes may form when a Fab galactose region inserts into the Fc' pocket left vacant by a galactose-deficient C α 2 oligosaccharide (118).

Idiotypes and Autoimmunity

Immunoglobulin (Ig) idiotypes are essentially phenotypic markers of the V genes used to encode Ig molecules either as soluble antibody molecules or as lymphocyte receptors. Idiotypes are therefore serologically defined determinants present in the Fab region of Igs, where they represent novel antigenic determinants which can be recognized in turn by anti-idiotypic antibodies.

Idiotypic anti-idiotypic reactions occur spontaneously in many autoimmune diseases including SLE (120), myasthenia gravis (121) and rheumatoid arthritis (122). Several investigators have demonstrated the occurrence of spontaneous autoanti-idiotypic antibodies directed at cell receptors following immunization with the receptor ligand. Wasserman et al (123), immunized rabbits with BisQ; a synthetic agonist of the acetylcholine receptor (AChR), to produce anti-BisQ antibodies. Those

antibodies were used to immunize a further group of rabbits which produced anti-idiotypic antibodies i.e. anti-(anti-BisQ) antibodies. It was found that the anti-idiotypic antibodies bound not only to the anti-BisQ antibodies but also to the AChR causing, in some animals, a myasthenia gravis like syndrome.

Equally, Shechter et al (124), found that mice immunized with bovine or porcine insulin produced both anti-insulin antibodies and autoanti-idiotypes that were anti-(anti-insulin) antibodies. The anti-idiotypic antibodies were reactive to the insulin receptor on fat cells where they partially mimic the action of insulin and block the binding of insulin itself. A similar effect is also seen in rabbits immunized with thyroid stimulating hormone (TSH). Anti-idiotypic antibodies to anti-TSH antibodies can mimic the thyroid stimulatory activity of TSH thereby causing Graves' disease (125). Recently, Zuberi et al (126) found that injection of mouse interleukin-1 (IL-1) into rabbits firstly produced anti-IL-1 antibodies and later anti-idiotypic antibodies which mimicked IL-1 activity by augmenting the in vitro proliferation of PHA-stimulated thymocytes.

Several investigators have proposed that autoantibodies per se, are anti-idiotypic antibodies produced against anti-microbial antibodies (127, 128). As such, they would carry the internal image of the microbial antigen and bind to the microbial receptor on cells. By use of an anti-idiotypic serum, a structure homologous to the idio type of anti-reovirus antibodies was identified on neurons (129). Thus, encephalitis caused by reovirus Type 3 infection, need not necessarily involve the direct interaction of virus on target cells, but an immunologic attack by anti-idiotypes on viral receptors.

Similarly, idiotypic complementarity has been suggested between rheumatoid factor (RF) and some anti-bacterial antibodies. Immunization of both mice and rabbits with affinity-purified human RF induces antibodies reactive both with a streptococcal peptidoglycan polysaccharide (PGPS) antigen and human RF. Thus, rheumatoid factor is either anti-idiotypic to anti-PGPS antibodies or it carries an internal image of a PGPS epitope (130). Conversely, anti-idiotypic antibodies to monoclonal RF were found in a patient with IgM RF gammopathy two weeks after a pneumococcal bacteraemia (131). Thus, it has been suggested that rheumatoid factors are anti-idiotypic antibodies against virus-induced anti-Fc receptor antibodies (132). Furthermore, myasthenia gravis has been reported to occur in 5 patients shortly after vaccination against rabies virus (133). The rabies virus binds to the AChR and thus a consequence of the anti-viral response is that some anti-idiotypic antibodies were anti-receptor, and invoked transient myasthenia gravis.

Autoantibodies may arise when cross-reacting idiotypes on the autoantibodies, appear on non-specific parallel immunoglobulin sets i.e. antibodies with the cross-reacting idotype but a differing antigenic specificity. Thus, following infection T helper cells with specificity for the cross-reacting idotype may be generated and provide helper activity for all B cells carrying the cross-reactive idotype to secrete antibody, some of which may be pathogenic. Connectivity between the AChR and -1,3-dextran (DEX) has been demonstrated, in that an anti-idotype from the DEX immune response was also an autoantibody against the AChR (134). Since the DEX determinant is present in some gut commensal organisms e.g. *Enterobacter cloacae*, there may be an idiotypic connection between antibody responses to the AChR and certain bacterial antigens.

An idiotypic relationship may also exist between anti-DNA antibodies bearing the common 16/6 idiotype and antibodies to Klebsiella (135). Patients with Klebsiella infections have an increased incidence of high 16/6 idiotype titres compared to other gram negative infections and normal controls. Furthermore, Klebsiella antigens can activate normal peripheral blood lymphocytes to secrete significantly increased levels of 16/6 idiotype compared to other polyclonal activators (136). This implies the 16/6 idiotype is present on antibodies binding Klebsiella antigens in non-autoimmune subjects and on autoantibodies to DNA in patients with SLE. Thus, autoimmune phenomena with pathologic sequelae may unfortunately develop following idiotype-(anti-idiotypic) interactions arising from the normal functioning of the idiotype network in response to endogenous or exogenous antigens.

Thus far, I have outlined some of the developments which have paved the way to elucidating current understanding of the aetiological factors contributing to autoimmunity and autoimmune disease. The evidence indicates that propagation of autoimmunity involves consideration of several elements including genetic, immunological and environmental factors. It is perhaps only when the "pieces" combine and the "mosaic" is complete that pathologic as distinct from physiologic autoimmunity ensues.

Having established this background information, I now intend to review the evidence which suggests that insulin-dependent diabetes mellitus is an organ-specific autoimmune disease.

CHAPTER 2 - TYPE 1 DIABETES : AN AUTOIMMUNE DISEASE

(Human Studies)

Type 1 or insulin-dependent diabetes mellitus, contrary to clinical impression, is a chronic disease (137) characterized by hyperglycaemia, ketosis and insulinopenia, necessitating the administration of exogenous insulin to sustain life. Epidemiologic studies indicate the incidence of IDDM varies between 7.7-30 per 100,000 population per year, with the peak incidence during adolescence (138). The majority of studies report that both sexes are equally affected but in some studies a slight preponderance of males in younger children is reported (139). Seasonal variations, with autumn and winter providing the largest numbers of new cases, suggest that viral infections may be important precipitating factors in the pathogenesis of IDDM (140).

Although a major life saving treatment, insulin therapy is still associated with reduced life expectancy (141), increased morbidity (142) and increased perinatal mortality rates, even in specialist centres (143). These factors clearly stress the importance of research into the aetiological factors associated with this disease, with the aim of developing protocols leading to prevention of the disease.

Major advances have been achieved over the past 15 years in improving our understanding of the pathogenesis of IDDM. The use of animal models and observations of various immune phenomena in IDDM patients have suggested an autoimmune pathogenesis for Type 1 diabetes.

AUTOIMMUNE ASSOCIATIONS IN IDDM

One of the earliest indications for the role of autoimmunity in IDDM was the observation that it often occurs in association with other endocrine diseases of suspected autoimmune character. Insulin-dependent diabetes mellitus was reported in 15% of patients with

Addison's disease, and in 7-10% of patients with Hashimoto's thyroiditis (144). Equally, other autoimmune endocrinologic disorders are 4-5 times more likely to occur among IDDM patients than the non-diabetic population, with autoantibodies against various endocrine cells, other than pancreatic cells, a common occurrence (145). The nature of increased susceptibility to autoimmunity in IDDM remains unexplained, however, a significant advance was the realisation of a link between IDDM and certain haplotypes of the MHC (146).

HLA ANTIGENS

Genes of the major histocompatibility complex known as the HLA system in man, and located on chromosome 6, encode for three major classes of proteins (147, 148): Class I genes code for the classic transplantation antigens (HLA-A, B, C); Class II genes (HLA-DP, DQ, DR); and Class III genes which code for some components of the complement system (C2, Bf, C4a, C4b). Class I and class II antigens are heterodimeric cell surface glycoproteins. Class I antigens are composed of a 44KD transmembrane glycoprotein which is noncovalently associated with the 12KD protein β_2 -microglobulin (which is encoded on chromosome 15).

The HLA-A,B,C molecules are polymorphic and are present on virtually all nucleated cells, where they function as targets for cytotoxic T lymphocytes (149). The class II antigens, which are also highly polymorphic, are involved in communication between cells that regulate the immune response (148). Class II antigens consist of two transmembrane glycoproteins with a light or beta chain (27-29KD) noncovalently bound to a heavy or alpha chain (33-35KD), both of which are encoded in the MHC. Class II antigens are normally restricted to the cell surfaces of B lymphocytes, activated T cells and antigen

presenting cells (APC) such as dendritic cells and cells of the macrophage/myeloid series (150). Therefore, each of these sets of antigens plays an important role in the control and functioning of certain aspects of immune responses.

HLA ANTIGENS AND IDDM SUSCEPTIBILITY

The early 1970's provided the first reports that demonstrated an association of IDDM with certain alleles of the HLA-B locus; namely, HLA-B8 and -B15 (11, 151). The use of mixed lymphocyte culture (MLC) typing established that a further stronger association existed with another locus in the HLA region, other than the B locus. Soon, the B locus association (due to linkage disequilibrium) was superseded by associations with the class II region genes, HLA-D/DR (152, 153).

In population studies, 90-95% of IDDM patients express the HLA-DR3 and/or DR4 haplotypes, with an excess of HLA-DR3/4 heterozygotes (154, 155). Indeed, the odds ratio or relative risk for the HLA-DR3/4 heterozygotes is greater than that for both the homozygous HLA-DR3 and HLA-DR4 patients (155, 156), suggesting the effects of HLA-DR3 and DR4 may be additive.

Studies in identical twin pairs where the concordance for IDDM is 30-50% (157, 158), have shown that the heterozygous HLA-DR3/4 phenotype was more prevalent in concordant pairs (59%) than with discordant pairs (28%)(159). In addition, a report by Knip et al (160) indicates that diabetic children who are HLA DR3/4 heterozygotes show a more rapid destruction of pancreatic beta cells than diabetic children who do not possess both DR3 and DR4.

Family studies also reflect the association of IDDM with particular MHC haplotypes (152). The prevalence of diabetes in parents of Type 1 diabetic children is about 10% (161), although IDDM is more likely in the progeny of diabetic fathers than diabetic mothers (161, 162). If one child has IDDM, the risk of diabetes in its siblings is related to the number of haplotypes the sibling shares with the diabetic proband (163). Thus, if two haplotypes are shared, the risk of developing IDDM is 10-20%; if one haplotype is shared it is 5%, and if neither haplotype is shared it is 1% (152, 163). However, if the shared haplotype is the heterozygous DR3/4, then the absolute risk of IDDM is 1 in 4 (164).

When this is compared with the absolute risk of IDDM for HLA-DR3/4 heterozygotes in the normal population being reported as approximately 1 in 40 (165, 166), then penetrance is stronger in familial than in sporadic cases and HLA typing is 6-10 times less powerful as a predictor of IDDM in the population than in affected families (166).

The existence of clinically distinct types of IDDM being associated with different HLA-DR phenotypes has been proposed (167). This is supported by the observations of a milder, more slowly progressing disease associated with HLA-DR3 (168); and as mentioned previously, the more rapid destruction of residual beta cell function in HLA-DR3/4 heterozygotes (160).

Whereas susceptibility to IDDM is associated with HLA DR3 and/or DR4, the presence of HLA-DR2 positivity is rarely found in patients with IDDM (about 2% compared with 30% in the normal population)(155), and the relative risk of IDDM in HLA-DR2 subjects is about 75 times less than that of the HLA-DR3/4 heterozygotes (146). Therefore, the presence of the HLA-DR2 gene or a gene in close linkage

disequilibrium with it, may protect individuals against IDDM.

Further serological and cellular typing analysis has subdivided the DR4 locus into five different subtypes (Dw4, Dw10 and Dw13-15)(170), of which only the Dw4 and Dw10 subtypes are associated with IDDM (171). Subsequent serological and DNA analysis identified the DQ3.2 allele of the DQB1 locus as a diabetes-associated subset of DR4 bearing haplotypes. Thus, approximately 95% of HLA-DR4 positive IDDM patients, but only 60-70% of DR4 positive controls, carry the DQ3.2 allele (172, 173).

When populations are screened for these markers, it is found that the extended haplotype profile increases the predictive value when compared with HLA-DR serology alone. Thus the absolute risk for subjects heterozygous HLA-DR3/Dw4-DQ3.2 or DR3/Dw10-DQ3.2 is increased to 1 in 15 (166). However, this is still less than the 1 in 4 risk in familial cases when HLA identical siblings share the DR3/4 haplotype, and therefore suggests that additional non-HLA genes and/or environmental factors are involved in the susceptibility to IDDM.

The usefulness of the above serological and genetic markers is limited by their high frequency in the normal population. Accordingly, another approach was to look for disease associated restriction fragment length polymorphisms (RFLPs) in the DNA of individuals with IDDM.

Studies using RFLP analysis have indicated that the HLA-DQ genes which are in linkage disequilibrium with HLA-DR, are more strongly associated with IDDM than the DR genes (172-176). Owerbach et al (174) was the first to describe a decreased frequency in HLA-DR4 and DR3/4 IDDM patients of an RFLP obtained with the restriction enzyme

BamHI, and identified with a DQ beta gene probe. The identified 3.7Kb fragment was frequent among HLA-DR4 and DR3/4 controls (30-40%), but was rarely detected in IDDM patients (0-2%). Studies by Cohen-Haguenauer et al (175) and Festenstein et al (176) have identified other DQ beta chain RFLPs, predominantly associated with the HLA-DR susceptibility to IDDM. Use of the restriction enzyme TaqI produced a band pattern designated DQR4 (175) with a high specificity for IDDM in HLA-DR3/4 heterozygote patients.

When calculating the relative risk for IDDM among HLA-DR3/4 positive subjects with this band, it was 10 times the relative risk, calculated on the basis of serologically defined markers alone (175). However, despite these associations no single RFLP has been found that is common to the several HLA haplotypes positively associated with IDDM.

Recently, Todd et al (177) have reported that the genetic susceptibility of IDDM associated with genes of the HLA-DQ locus is mainly dependent on the identity of a single amino acid residue at position 57 of the DQ beta chain.

As mentioned previously, about 95% of IDDM patients carry the HLA-DQ3.2 allele whereas another DQ allele (DQ3.1) is negatively associated with IDDM (173). Comparison of the amino acid sequences revealed that the DQ3.1 allele DQ beta chain first domain sequence had four amino acid differences from the DQ3.2 allele beta chain at positions 13, 26, 45 and 57. It was inferred that one or more of these four beta chain amino acid substitutions accounted for the positive association with IDDM of the DQ3.2 allele relative to the DQ3.1 allele.

Todd and colleagues cloned and sequenced the gene segments encoding the first domains of the four major expressed polymorphic class II gene products (HLA-DQ beta, DQ alpha, DR beta I and DR beta II). All sequences found in IDDM patients were also found in normal non-diabetic controls, indicating that IDDM susceptibility is not due to mutant HLA class II alleles. However, it was demonstrated that susceptibility to IDDM was conferred by the presence or absence of aspartic acid (Asp) at position 57 of the N-terminal B1 domain of the HLA-DQ beta chain. Thus, all the DQ beta alleles that were positively associated with IDDM had either alanine, valine or serine residues at position 57. Aspartic acid was present at position 57 in all the DQ beta alleles which were neutral or negatively associated with IDDM. Accordingly, the possession of two Asp-57-positive DQ beta alleles provides almost complete resistance to IDDM, whereas DQ beta Asp-57-negative homozygosity confers maximum susceptibility (90% of IDDM patients), and Asp-57 heterozygosity carries a much lower risk of developing IDDM (10% of IDDM patients).

The mode of inheritance for IDDM is unknown, but is clearly polygenic (178). It is proposed that at least two genes; one associated with HLA-DR3 and one with HLA-DR4, but not necessarily these genes themselves, are required for diabetes to develop (137). It has been estimated that 50% of the heritability of IDDM is contributed by genes within the HLA-D region (179), which allows consideration for the involvement of non-HLA genes.

Consequently, recent studies have reported the association of IDDM with RFLPs at a locus near the insulin gene located on the short arm of chromosome 11 (180, 181, 182). Equally, susceptibility to IDDM may be linked with DNA polymorphisms of the switch region of

immunoglobulin heavy chain genes (183) or genes encoding the T cell receptor (184).

The precise role of the MHC class II gene products in determining the susceptibility to IDDM is not known. It is widely accepted that the MHC class II genes determine the host immune responsiveness to foreign antigens (150). Thus, individuals with different HLA-DR haplotypes can respond differently to identical antigenic stimuli (185). The MHC class II gene associated susceptibility to IDDM, is assumed to reflect differences between the different HLA-D region haplotypes in their ability to recognize and present antigen to the various subsets of T lymphocytes. Thus, Todd et al (177) suggest that the difference between diabetes susceptible (DQ beta Asp-57-negative) and diabetes resistant (DQ beta Asp-57-positive) individuals, is that the "diabetogenic" peptide cannot effectively bind into the Asp-57-positive MHC class II containing groove. Thus failure of antigen presentation or inadequate presentation of the appropriate antigen is assumed to occur in the diabetes resistant individuals.

An alternative proposal by Nerup et al (146) suggests that specific "qualitative" IDDM genes do not exist, but rather the HLA associations with IDDM reflect "quantitative" differences whereby the different class II genes control different levels of cytokine production, and thereby influence the magnitude of the immune response.

In conclusion, although possession of certain HLA haplotypes appears linked with an increased propensity to develop IDDM, mere possession of the DR3 and/or DR4 haplotypes is not sufficient for development of IDDM. Accordingly, 50-55% of the normal population also carry the HLA-DR3 and/or DR4 haplotypes and do not develop IDDM thereby suggesting the involvement of non-HLA genes

(186). Furthermore, the low concordance rate of IDDM between identical twins (157, 158) suggests that exogenous (environmental) factors play an important role in the manifestation of Type 1 diabetes.

HUMORAL IMMUNITY

Further indications for an autoimmune component in the pathogenesis of IDDM shortly followed the initial reports of HLA-IDDM associations, when autoantibodies directed towards pancreatic islet cells were detected in the sera of IDDM patients.

Islet Cell Cytoplasmic Antibodies

In 1974 the first reports were made on the presence of circulating cytoplasmic islet cell antibodies (ICA) in IDDM patients. In the initial reports (187, 188), ICA were found in patients with coexistent polyendocrine disease, but subsequently were found in most IDDM patients at onset of disease (189). Islet cell antibodies are detected on cryostat sections of human pancreas from blood group O donors (this is to prevent interfering staining of the acinar tissue due to expression of blood group antigens), with standard indirect immunofluorescence techniques. Islet cell antibodies are found in 0.5% of the normal population, 3% of healthy first degree relatives of ICA positive subjects, 10% of NIDDM patients and in 6% of patients with other organ-specific autoimmune diseases (189, 190). The prevalence of ICA is relatively high (70-90%) in newly diagnosed IDDM patients (189, 191). The frequency of ICA decreases with the duration of IDDM such that only 20% of patients remain positive after 2-5 years (192). This probably reflects the gradual loss of antigenic

stimulus, as islet cell destruction progresses to a total absence of beta cells.

Sera positive for ICA react not only with the cytoplasm of islet beta cells, but with all islet endocrine cell types (glucagon, somatostatin and pancreatic polypeptide (PP) cells). Neither the subcellular localization of the antibody binding site nor the nature of the antigen(s), to which ICA react, have been determined. However, the antibody activity is not absorbed with insulin or glucagon (191) and some evidence suggests that a sialic acid containing glycolipid may be involved (193).

Since only beta cells are depleted in the course of developing IDDM (194), ICA, because of their broad cell specificity, are unlikely to play a direct role in beta cell destruction. Indeed, ICA do not show cytotoxicity towards cultured rodent islets (189) and in a study documenting the transplacental passage of ICA from diabetic mothers to their offspring, ICA did not contribute to any clinical or metabolic alterations observed in the infants (195). It is likely that ICA develop secondarily as a response to islet cell damage and, therefore, may be useful as a marker of ongoing beta cell destruction and hence have some potential for predicting future IDDM patients.

The prognostic value of ICA has been ascertained by several groups. Irvine et al (196) showed that ICA positivity was a predictor for future development of IDDM in diabetic patients maintained with oral hypoglycaemic agents. Others have reported the predictive value of ICA positivity in gestational diabetes where, in one study, 73% of gestational diabetics who were ICA positive subsequently developed overt IDDM within 10 years, while none of 12 ICA negative patients became insulin-dependent (197). Studies on families of patients with IDDM,

indicate that ICA may be present several years prior to the onset of clinical diabetes thereby suggesting a long latent or prediabetic period (198, 199).

In one study 38 of 135 subjects, at high risk of developing IDDM, were ICA positive and 14 subsequently progressed to diabetes after one month to several years (200). In a further prospective study of a set of monozygotic triplets and a set of monozygotic twins initially discordant for IDDM, ICA preceded the onset of overt diabetes in the twin by 5 years and by 8 years in one of the two remaining non diabetic triplets (201).

However, the predictive value of ICA alone is limited. Islet cell antibody positivity was only followed by IDDM in about 30% of ICA positive unaffected first degree relatives of IDDM patients followed for 5 years (202). Thus, the false positive rate is relatively high. Equally, IDDM can occur in ICA negative subjects (203) and ICA may appear and disappear in non diabetic individuals (202), although the latter is unconfirmed in other studies (204).

Therefore, current opinion would suggest that ICA develop in response to beta cell injury and, as such, represent a marker of ongoing beta cell destruction, but are not ultimately predictive of impending Type 1 diabetes. Equally, their lack of specificity suggests that they are not pathogenetically involved in the initiation of beta cell damage.

Reports of a separate variant of ICA which can fix complement, stimulated a revived pathogenetic importance for ICA (205). Complement-fixing ICA (CF-ICA) are also detected by indirect immunofluorescence on pancreatic tissue sections. It is generally found that about 50% of conventional ICA are also complement fixing, but

complement fixation does not occur without the presence of cytoplasmic ICA (206). However, at onset, about 80% of cytoplasmic ICA may also be CF-ICA. On follow up, over 1-18 months, about 35% of patients lose the complement fixing capacity (205).

Since CF-ICA appeared more closely related to the onset of clinical disease; more specific for beta cells and disappeared more quickly than conventional ICA, it was suggested that CF-ICA more selectively reflected the damage of pancreatic beta cells and, therefore, are a better predictive marker than ICA (205).

In a study reported on 13 patients with polyendocrine disease and ICA, 9 of the patients were also CF-ICA positive, of whom 4 subsequently became diabetic within 5 years (207). The value of CF-ICA is however uncertain, since several studies indicate that CF-ICA merely represent high titred conventional ICA and are not a separate species of ICA (208, 209).

Islet Cell Surface Antibodies

In 1975, MacLaren and co-workers found antibodies reactive with the surface of cultured human insulinoma cells in 34 out of 39 diabetic patients (210). Subsequently, Lernmark et al (211) described the presence of antibodies to the plasma membranes of islet cells; islet cell surface antibodies (ICSA) in the sera from newly diagnosed IDDM patients. These antibodies, as with ICA, were detected by indirect immunofluorescence. Later, quantitative analysis of ICSA, using radiolabelled protein A, was reported (212). Islet cell surface antibodies can also be detected using cultured rat insulinoma cells (213) and human foetal (214) or adult (215) islets. Islet cell surface antibodies are found in 70-80% of newly diagnosed IDDM patients and subsequently

decrease with increasing duration of diabetes (216-218). Although ICA and ICSA are both usually present concurrently (218), they do not always correlate (216).

Unlike ICA, ICSA appear preferentially beta cell specific (218-221). In a study by Van De Winkel et al (221), 21 out of 38 diabetic patients were ICSA positive. Subclasses of ICSA, with varying degrees of specificity for the different islet cell types, were identified.

Whereas sera from ICSA patients aged under 30 years were found to bind exclusively to beta cells, ICSA positive serum from a few older diabetics preferentially bound to glucagon and PP cells. Equally, ICSA are not exclusive to IDDM, being found in Type II diabetes (221) and in 30% of nondiabetic patients with autoimmune thyroid disease (211).

The prognostic value of ICSA is unclear, since an unacceptably high proportion (25%) of healthy first degree relatives are also positive for ICSA (218), whereas only 5% will ultimately develop IDDM. In addition, no correlation was detected between ICSA status and insulin secretion after an oral glucose load in nondiabetic relatives of IDDM probands (222).

Serum positive for ICSA obtained from children with recently diagnosed diabetes, were found to immunoprecipitate a 64 kilodalton (KD) protein obtained from human islets (223). Antibodies recognizing the 64KD islet protein may be detected up to 8 years prior to the onset of IDDM (224). However, it is not known to what extent the 64KD protein is specific for islet beta cells.

Islet cell surface antibodies in contrast to ICA may have some pathological importance in the development of IDDM. In the presence of complement, ICSA positive sera can

cause lysis of cultured islets (218-220, 225). Using a double fluorescence technique, that identified lysed cells by their capacity to take up ethidium bromide and beta cells by their staining with a fluorescein-conjugated antibody to insulin, it was shown that ICSA complement-dependent cytotoxicity was preferentially directed towards beta cells (218, 219), although approximately 10% of non-beta cells were also lysed (219). Equally, treatment of islet cell cultures with ICSA positive serum and complement, inhibited glucose stimulated insulin release (220, 226), but arginine stimulated glucagon release was unaffected (220). Furthermore, in the absence of complement, perfusion of dispersed rat islet cells with purified IgG from ICA, ICSA and anti-64KD positive sera has been shown to also inhibit glucose induced insulin release (227).

Islet cell surface antibodies may mediate cytotoxicity via antibody dependent cellular cytotoxicity (ADCC) whereby ICSA bind to the Fc receptors on cells such as macrophages, K and NK cells. Accordingly ADCC of lymphocytes and serum from diabetic patients directed against human insulinoma cells was described (228). In addition, increased K cell levels have been reported in newly diagnosed IDDM patients (229, 230). However, this represented non-specific ADCC directed against human erythrocytes and was also found in unaffected siblings at comparable levels (230). Specific ADCC directed towards rat islet cells has been reported, however, there was no significant increase in ADCC activity amongst IDDM patients (231).

Although ICSA precede the onset of IDDM, and in vitro exert complement-dependent cytotoxicity towards cultured islet cells, 25% of first degree relatives also show ICSA positivity with accompanying beta cell cytotoxicity but without diabetes (218). Furthermore, the presence of

ICSA in 2 out of 4 subjects, in whom diabetes developed following ingestion of the rodenticide "Vacor" (232), suggests that as with ICA, ICSA develop following beta cell injury and therefore represent a further marker of ongoing beta cell destruction.

Insulin Autoantibodies

Autoantibodies against insulin have been reported in some rare cases of hypoglycaemia in patients which had not been treated with insulin (233, 234). More recently, Palmer et al (235) described the presence of circulating insulin autoantibodies (IAA) in children at diagnosis of IDDM. In this initial report; using a liquid phase radio-immuno-assay (RIA) and a porcine insulin tracer, 18% of children at diagnosis were positive for IAA and IAA were not detected amongst the siblings of the diabetic probands. Insulin autoantibodies that develop spontaneously must be distinguished from insulin antibodies that occur following injection of exogenous insulin and, as such, are common amongst insulin treated IDDM patients. Following the initial report by Palmer and colleagues, several other groups have confirmed the presence of IAA in the sera of newly diagnosed IDDM patients (236-240). However, controversy persists amongst the different centres regarding the prevalence and the predictive value of IAA as a marker for IDDM. To a large extent, this is due to the use of different assay systems (enzyme-linked immunosorbent assay (ELISA) versus RIA) and the use of different insulin species (human versus porcine or bovine) as substrate. However, as a consensus, 20-40% of IDDM patients, at onset, are positive for IAA (236-239) and IAA can be detected several years prior to the onset of IDDM (241-243).

Accordingly, some groups have reported a correlation between IAA and ICA (238, 242, 243), although other groups do not confirm this association (236, 237, 239). Equally, some reports document an association between IAA and HLA-DR3 or DR4 haplotypes (238), whereas others do not (237, 239).

Further discrepancies have been reported regarding the frequency of IAA amongst discordant identical twins. In one study, the overall frequency of IAA amongst discordant twins was 47% (239); furthermore, 14 out of 39 twins which were ICA negative were positive for IAA. This contrasts with another study in which IAA were present in only 1 of 22 ICA negative twins (242).

Since IAA were found in 7 of 14 nondiabetic twins of longstanding diabetics (duration of discordance 15 years or more)(239), by which time the risk of developing diabetes is only about 3% (158), the pathogenetic importance of IAA would appear minimal and the prognostic value of IAA when considered singularly is poor. However, if IAA is evaluated in conjunction with other islet cell antibodies, the combined presence of several autoantibodies within the same subject represents a higher predictive index than the presence of any marker alone (238, 242, 243). Thus, in studies on beta cell function, insulinopenia following intravenous glucose stimulation was present in 70% of ICA positive IAA positive subjects, compared to 23% of ICA positive, IAA negative individuals (238).

Other Autoantibodies

In addition to IAA, autoantibodies against the insulin precursor; proinsulin, have been reported. In a study where only IAA negative subjects were screened, proinsulin autoantibodies (PAA) were present in 21% of



recent onset IDDM patients (244). Inhibition of PAA binding was achieved by the addition of proinsulin or C-peptide, but not with insulin.

Diabetes incurred by insulin resistance following anti-insulin receptor antibodies impeding the binding of insulin with its receptor is associated with the rare condition acanthosis nigricans (245). Although anti-insulin receptor antibodies have been reported in 40-45% of newly diagnosed IDDM patients (246, 247), the severe insulin resistance associated with acanthosis nigricans is absent. In some IDDM patients, anti-insulin receptor antibodies may represent anti-idiotypic antibodies to anti-insulin antibodies (124, 248). The anti-idiotypic or insulin receptor antibody may be formed following release of insulin antigens from damaged beta cells and, as such, provide another marker of ongoing beta cell destruction.

Aside from the specific humoral autoimmunity aimed towards the islet cell, several studies show a general hyperactivity of the humoral immune system amongst IDDM patients.

An increased incidence of autoantibodies against thyroid, adrenal and parietal cells, was found in children with IDDM (145, 249) and their first degree relatives (250). The presence of multiple autoantibodies in IDDM patients is in accord with the demonstration of polyclonal B cell activation in recent-onset IDDM (251). Furthermore, 7 out of 9 human hybridomas prepared from peripheral blood lymphocytes, from patients with IDDM, were found to react with antigens present in multiple endocrine organs (anterior pituitary, gastric mucosa, thyroid and pancreatic islets), reflecting a common antigenic determinant amongst these organs (252).

Finally, as is common in other autoimmune conditions, lymphocytotoxic antibodies are found in the sera of IDDM patients (253). The parallelism of these antibodies occurring in different autoimmune diseases, suggests that they are part of a generalized immune response of autoimmune diseases.

In summary : Various parameters of humoral immunity have been described amongst IDDM patients and their first degree relatives. Islet cell antibodies and ICSA are detectable several years prior to the onset of IDDM and ICSA can participate in complement-dependent, antibody mediated, cell cytotoxicity to xenogeneic islet cells in vitro. Nevertheless, these antibodies are also present in healthy subjects without causing disease and therefore, must be considered of limited value as predictive markers of IDDM. Equally, since IAA, which carry minimal pathogenetic potential, can be detected as early as ICA or ICSA in the prediabetic period, the mere presence of any of these antibodies is not indicative of a pathogenetic role. Indeed, most islet cell antibodies are not beta cell specific and no one has been able to transfer IDDM from affected to unaffected individuals using any of the islet cell directed antibodies; although one report describes a lower glucose stimulated insulin release in immunosuppressed mice treated with IgG from diabetic children as compared with mice injected with IgG from non diabetic children (254).

In general, most evidence suggests that anti islet cell antibodies result from polyclonal B cell activation following release of islet antigens from damaged beta cells, and therefore are probably of secondary pathogenetic importance. As such, they represent markers of beta cell injury but not necessarily impending IDDM. However, it is possible that they may be involved in the later stages of beta cell destruction.

CELLULAR IMMUNITY

Histopathology

The characteristic pathological finding in the pancreata of IDDM patients, at onset of disease, is that of mononuclear cellular infiltration within the pancreatic islets. Von Meyenburg termed this infiltration "insulitis" (255) and insulitis accompanied by selective beta cell destruction (256) are the pathological hallmarks of Type 1 diabetes (194, 257-259). It has been estimated that approximately 90% of all beta cell mass has to be destroyed before the clinical signs of IDDM are manifest (257). The actual frequency of insulitis remains a contentious issue.

In a 25 year review of deaths in patients aged under 20 years, insulitis was present in 47 of 60 patients with recent onset disease (259). In a study by Gepts (257), islets from 16 of 23 patients with recent onset IDDM revealed insulitis. In another study, 6 of 11 patients who died within 2 months of the onset of IDDM, presented with insulitis (258). However, Doniach and Morgan failed to observe insulitis in any of 13 patients with recent onset disease (260). The extent of insulitis within individual IDDM patients is also variable, affecting 13-75% of islets and is more pronounced among young patients. (258).

Many of the discrepancies over the frequency and extent of insulitis are due to the heterogenous pathology of IDDM. Gepts (257) reported large areas of the pancreas showing only inconspicuous pseudoatrophic islets i.e. islets devoid of beta cells and insulitis. In other pancreatic lobules, many large islets, predominantly composed of hyperactive beta cells, were found. Some of these islets were free from insulitis, whereas others contained only a few infiltrating lymphocytes. But, in

some partly shrunken islets, lymphocytic infiltration was found along with a reduced proportion of beta cells. Thus, tissue sampling from a diseased organ with such a heterogenous and patchy pathology will obviously give rise to discrepancies between different studies. However, in most cases, insulitis is observed only in islets still containing insulin (259), indicating it disappears once the beta cells are destroyed. Consequently, insulitis is rarely found in IDDM patients with a disease duration longer than a year (259).

In one patient with recent onset IDDM, who died within 24 hours of diagnosis, immunofluorescence studies were performed to characterize the phenotypes of the infiltrating cells (261). Most of the infiltrated cells were CD8⁺ T cytotoxic/suppressor (T cyt/supp) cells, although CD4⁺ T helper (T_H) cells, NK cells and B lymphocytes were also present. Furthermore, T cells, mainly T cyt/supp, as well as macrophages contributed to the insulitis found in long term IDDM patients with recurrent disease having received segmental pancreatic transplants from their discordant identical twins (262).

Cell- Mediated Immunity

In 1971 Nerup et al (12), using the leucocyte migration inhibition test and a homogenate prepared from pooled porcine pancreas, first demonstrated the presence of lymphocytes with reactivity for pancreatic antigens in the circulation of recently diagnosed IDDM patients. Moreover, a subcutaneous injection of pancreatic homogenate into 4 of 6 patients, whose leucocytes were inhibited in the migration test, led to a Type IV hypersensitivity reaction, indicating that the in vivo assay reflected cell-mediated immunity (CMI) towards the pancreas. Leucocyte migration inhibition activity accordingly decreases with increasing disease duration.

Huang and MacLaren (228), using a dye exclusion test to determine cell death, were the first to report that lymphocytes from acutely diabetic children showed increased cytoadherence and cytotoxicity towards a human insulinoma cell line. Boitard et al (263) reported that mononuclear cells from 80-90% of IDDM patients inhibited glucose stimulated insulin release from mouse islets in vitro. The inhibition was specific for insulin since arginine stimulated glucagon release was unaffected (264). It was further noted that the OKT3⁺ OKT4⁻ E rosette forming cells were responsible for mediating this effect (264).

The passive transfer of diabetes with specific effector lymphocytes from a diseased donor to a healthy recipient, in accordance with Witebsky's criteria for autoimmune diseases (22), has not been achieved. Buschard et al (265) reported elevated blood glucose levels in athymic nude mice which received intraperitoneal injections of peripheral blood lymphocytes derived from newly diagnosed IDDM patients. However, other investigators have been unable to confirm this finding (266, 267). Furthermore, results from passive transfer studies involving allogeneic or xenogeneic transfer require consideration of possible GVHD, which has been shown to cause insulinitis (268).

The importance of CMI in the pathogenesis of IDDM was highlighted in the evaluation of pancreatic transplants between identical twins. Sutherland et al (269) transplanted the tail of the pancreas from 3 normal monozygotic twins who were ICA negative, into their diabetic twins, all of whom had been diabetic for over 15 years. Since the twins were identical, it was deemed unnecessary to give immunosuppression.

Initially the grafts reversed the diabetic condition, but within a few weeks the islets in the pancreatic grafts became infiltrated, predominantly by activated T lymphocytes; beta cell destruction followed and diabetes recurred (262, 269). In a fourth patient, who received a similar transplant but also received immunosuppression with azathioprine, insulinitis or diabetes was not recurrent (269).

Circulating Lymphocytes and Abnormal Immunoregulation

Studies of circulating lymphocytes in IDDM patients have yielded conflicting results. This may be accounted for by the use of different assays and the selection of patients at different stages of disease. Equally, hyperglycaemia and ketoacidosis per se can have effects on lymphocyte function and distribution (270, 271). Nevertheless, in some studies normal total T and B lymphocyte numbers have been found regardless of the degree of glycaemic control (272, 273). Other studies have documented a decrease in T lymphocytes that were either dependent (274) or independent (275) of the level of glycaemia.

The use of monoclonal antibodies to perform lymphocyte subset analysis has provided a further spate of conflicting studies. Horita et al (276) reported that the ratio of helper/suppressor cells was increased in IDDM patients of disease duration less than 2 months, which then later returned to normal. Ilonen et al (277) however reported a decrease in the ratio of helper/suppressor cells. Most studies are agreed that the frequency of T_H cells in IDDM patients at onset of disease are normal (277-279). However, T cyt/supp cells are variously reported as normal (278, 280), increased (272, 277) or decreased (273, 279).

Several studies report an increase in the number of activated T cells in the circulation of recently diagnosed IDDM patients (272, 277, 278, 280-282). This phenomena was first described by Jackson et al (278); in which 9 of 11 diabetic patients were found to have approximately 10% of their circulating T lymphocytes bearing MHC class II or Ia antigens, whereas controls had 1% Ia-positive T cells.

In another study, raised levels of HLA DR expressing T cells were found in 14 of 15 recently diagnosed diabetics, but in only 7 of 28 patients with disease duration longer than 3 years (281). De Berardinis et al (282), further describes the presence of IL-2 receptor (IL-2R) bearing T cells in 13 of 25 recently diagnosed IDDM patients. Furthermore, they demonstrated that most of the activated T cells were confined to the CD4 population of cells (282). The CD4 population has been subdivided into two groups; helper-inducer (CDw29), which are the helper cells for antibody production (283) and suppressor-inducer (CD45R) cells (284). In other autoimmune disease such as SLE (285) and multiple sclerosis (286), immunoregulatory disturbances with decreased numbers of suppressor-inducer cells are found. However, in IDDM patients there was no decrease in either of the CD4 subsets, and the increase in IL-2R positive cells was present in both populations of CD4 cells (282).

The presence of increased numbers of Ia-positive T cells at onset of IDDM, suggested that this may prove useful as a predictive marker for IDDM. Accordingly, Alviggi et al (281) reported increased levels of activated T cells in 5 of 7 unaffected co-twins of recently diagnosed IDDM patients. In two of the twins, retested 6 months later, the raised levels of HLA DR expressing T cells persisted and impaired glucose tolerance had developed. Equally, increased numbers of Ia-positive T cells were reported in

5 of 10 ICA positive normoglycaemic siblings of IDDM patients (272).

Although disturbances in the cellular composition of peripheral blood lymphocytes from IDDM patients have been reported, it is necessary to emphasize that these changes may not be a true reflection of the pathologic processes occurring in the islets of Langerhans. However, in one study, peripheral blood lymphocytes from 5 patients with recent onset IDDM, were isolated, radiolabelled and autologous transfusions performed by reinjecting the lymphocytes into the same patient from which they were removed (287). Using an emission-computerized scanner, radiolabelled lymphocytes were detected in the vicinity of the pancreas in 3 of the 5 patients.

Numerous studies have investigated the functional status of lymphocytes from IDDM patients. Natural killer cell activity is reportedly decreased (288), although K cell activity is increased (229). Several in vitro studies have suggested that suppressor T cell activity in recent onset IDDM is decreased (289-292). However, abnormal T suppressor cell function has also been demonstrated in non diabetic subjects who are HLA DR3 (293) and in approximately 20% of healthy first degree relatives of IDDM patients (294), suggesting that decreased suppressor cell activity may be an abnormality related to genetic background. Equally, altered suppressor cell activity may be a consequence of the disturbed carbohydrate metabolism of IDDM patients (292).

Deficient production of IL-2 has been demonstrated in several autoimmune disorders (295), including Type 1 diabetes (296, 297). However, in IDDM patients the deficiency represents an acquired defect in response to beta cell destruction, rather than a precipitating factor of IDDM (297). The decreased IL-2 production is

unrelated to the degree of glycaemic control and, unlike other autoimmune phenomena found in IDDM patients, appears to persist for several years after the onset of disease (297).

In conclusion : Many of the studies herein described suggest a strong role for cell-mediated immunity in the pathogenesis of Type 1 diabetes mellitus. However, as mentioned in the case of humoral immunity, there have been no reports of the successful transfer of diabetes using peripheral blood lymphocytes derived from IDDM patients. This indicates the requirement of an initial triggering stimulus prior to the activation of autoreactive lymphocytes. Phenotypic analysis of circulating lymphocytes in IDDM patients have provided variable results which, in any case, may not be representative of the intra-islet events leading to the development of IDDM. Phenotypic characterization of the insulinitis has identified a variety of infiltrating cells including T cells (T cyt/supp and T_H), B lymphocytes, NK cells and macrophages. The difficulty lies in distinguishing which (if any) of these cells are responsible for directing the demolition of beta cells; and those which are merely innocent bystanders. Furthermore, these studies are performed at a stage of disease development when more than 90% of the insulin-producing beta cells are destroyed and, as such, give little information on the early initiating events of the prediabetic period.

Thus far, I have described a variety of evidence which suggests that autoimmune phenomena are involved in the pathogenesis of Type 1 diabetes mellitus. Since IDDM is still associated with increased morbidity and mortality rates, the ultimate aim of diabetes research is to prevent the disease.

The development of a rational therapeutic approach to prevention, depends however on (i) the identification of accurate markers for the development of clinical disease, as distinct from genetic susceptibility, (ii) elucidation of the precise sequence of events leading to mass (more than 90%) destruction of islet beta cells, and (iii) detailed examination of the immediate and long-term effects of possible therapeutic intervention.

Obvious difficulties in studying these areas, in man, include the practical difficulties involved in conducting longitudinal prospective studies of genetically susceptible cohorts. Furthermore there are the difficulties in correlating cellular and humoral immunity with pancreatic morphology and function, and the inability to standardise and control genetic factors in a condition whose clinical expression is determined by the interaction of genetic susceptibility and environment.

Finally, there may be ethical problems for the application of certain intervention studies. This dictates the need for studies using animal models to precede and complement those involving the human IDDM patient.

**CHAPTER 3 - THE BB RAT AND OTHER ANIMAL MODELS
OF TYPE 1 DIABETES MELLITUS**

Numerous animal species displaying either spontaneous or induced hyperglycaemia have been described (298). This thesis will describe studies performed on one such animal model; the spontaneously diabetic BB rat. In addition, reference will also be made to two other commonly used animal models of IDDM, namely the non-obese diabetic (NOD) mouse and multiple low-dose streptozotocin-induced diabetes of mice.

THE SPONTANEOUSLY DIABETIC BB RAT

The spontaneously diabetic BB rat was discovered serendipitously in 1974 by Drs Reginald and Clifford Chappel in a colony of albino Wistar rats, maintained at the BioBreeding Laboratories (from which the BB designation is derived) in Ottawa, Canada (299).

In 1977 a breeding colony of BB rats was established at the Animal Resources Division of Health and Welfare in Canada. This colony subsequently supplied animals to establish other colonies including our own in Edinburgh. In accord with the recommended nomenclature, the original colony is designated BB, the colony in Edinburgh BB/E, that at Worcester BB/W and so on (302).

Particularly attractive features of this animal model, as a research tool for work in IDDM, include a prediabetic period of about 3 months; involvement of both genetic and immunological factors in the aetiology; the fact that not all animals in diabetes-prone (DP) litters develop clinically overt insulin-dependent diabetes; the absence of obesity; and the occurrence of functional and structural changes in the retina, kidneys and nerves of established diabetic animals.

The main disadvantages of the model relate to its increased susceptibility to infection (300); difficulty in animal care and breeding; the need to create, by long term selective breeding, a subline of diabetes-resistant (DR) BB rats to serve as controls, so that it can be determined whether any observed changes are diabetes rather than strain related. It is important to emphasize that it is inappropriate to use non diabetic littermates of animals with IDDM as normal controls, since many of these animals have abnormal blood glucose profiles, whilst not requiring insulin to survive (301). In addition, the incidence of diabetes among DP litters varies widely not only between colonies, but also within the same colony, where inadvertent environmental changes may influence the incidence of diabetes. Environmental factors identified as affecting the incidence of diabetes include diet (303), stress (304) and infection (305).

GENERAL CHARACTERISTICS

The first papers describing the metabolic and morphologic characteristics of the diabetic syndrome in the BB rat, were from Nakhoda et al (301, 306, 307). Unlike the non-obese diabetic (NOD) mouse model, where diabetes preferentially affects females (308), diabetes in the BB rat equally affects both sexes. Diabetes in the BB rat occurs between 60-140 days of age, coinciding with rat puberty, although it is not uncommon for diabetes to develop later in some animals.

The BB rat displays all the clinical symptoms characteristic of the human disorder including polyuria, polydipsia, glycosuria and severe weight loss, despite hyperphagia (306). Diabetic animals succumb from ketoacidosis within 2 weeks of onset, unless exogenous insulin is administered. At onset, hyperglycaemia is associated with hypoinsulinaemia, hyperglucagonaemia,

hyperketonaemia, hyperlipidaemia and uraemia. Blood levels of branched chain amino acids and taurine, glycine, tyrosine, phenylalanine, ornithine and lysine are all raised, while alanine and lactate are decreased (309). Increased serum levels of 3-methylhistidine (reflecting muscle protein breakdown) together with enhanced ammoniogenesis suggest a generalized catabolic state (310).

Both glucose tolerance and the clinical expression of diabetes, correlate with the insulin response to a glucose challenge, which in turn correlates with the presence of insulinitis (311).

The degree of insulinitis varies between animals, but always precedes overt hyperglycaemia and corresponds to the severity of the diabetic symptoms (312). Thus, mild patchy insulinitis commonly exists without any detectable impairment of either glucose tolerance or insulin secretion. Other more severe and extensive insulinitis is associated with impaired glucose tolerance and reduced glucose-induced but not arginine-induced secretion of insulin (313), with first phase insulin being particularly affected (537). At onset, massive insulinitis and a decrease in the number and size of pancreatic islets is found (312). However, progression through these phases is not inevitable (301) and whilst IDDM never occurs without insulinitis, animals with insulinitis do not always develop IDDM.

The BB rat appears very susceptible to the development of pulmonary and other infections by common varieties of bacteria, mycoplasma and viruses (314), and to the development of lymphoproliferative disorders (315). This may be related to the lymphopenia commonly, but not invariably, seen in these animals. Initially it was thought that this was mandatory for the development of

IDDM (316, 317) and since lymphopenia is not a feature of human IDDM, this appeared to represent a major difference between IDDM in man and the BB rat model. However, it is now established that not only is lymphopenia frequently present without diabetes, but also that IDDM can develop in the absence of lymphopenia (318, 319).

Organ system changes suggestive of human diabetic complications are also found in well established insulin treated diabetic BB rats. Although the classical clinical features of diabetic retinopathy are not seen on ophthalmoscopy in the BB rat; retinopathology including pericyte degeneration and loss, endothelial cell degeneration and basement membrane thickening, have been described (320).

Diabetic neuropathy is the most common complication of diabetes and altered nerve conduction and morphological changes in somatic peripheral and autonomic nerves, characteristic of human diabetic neuropathy, are reproduced in the BB rat (321, 322). As with retinopathy, the pathological and clinical features of severe nephropathy found in human IDDM are not observed in the BB rat, although proteinuria and thickening of the glomerular basement membrane occur after a few months of diabetes (323).

Alterations in hepatic metabolism (324) and gonadal dysfunction (325) have also been reported. Although changes in myocardial biochemistry have been reported (326), atherosclerosis and severe microangiopathy, which are common in human IDDM, have not been found in the BB rat (327).

As is the case with human IDDM, where other endocrine glands are sometimes also affected, thyroiditis is found in some BB rats (328, 329) but occurs independently of

insulinitis and diabetes (329) and is not of sufficient severity to cause thyroid failure (328). The incidence of thyroiditis can, however, be increased by the administration of dietary iodide (330).

Thus it can be seen that many features of diabetes in the BB rat closely resemble those found in human IDDM patients. Many of the immunological parameters prevalent amongst IDDM patients described in Chapter 2 are also found in the BB rat. Indeed, assuming that diabetes in the BB rat is reflective of IDDM in man, much of the evidence obtained from studies on the BB rat has further substantiated and propagated the involvement of autoimmunity in the aetiology of IDDM.

GENETICS

Susceptibility to IDDM in the BB rat, as with human IDDM, is linked to a gene or gene cluster located within the rat MHC (331), although several non-MHC modifying genes affect the frequency and time of disease onset (332).

The rat MHC, known as RT1, resembles the mouse MHC (H-2) in that the class II loci (RT1B and RT1D) are flanked by the class I RT1A locus and the class I RT1E and RT1C loci (333). As with the Wistar Furth (WF) rat, from which it is derived, the BB rat carries the RT1^u haplotype.

A series of breeding studies (331, 334, 335) determined the importance of the u haplotype for the development of IDDM. Initially BB diabetic males were crossed with females of the inbred Lewis (Lew) strain (RT1^l) or Buffalo (Buf) strain (RT1^b). In the initial F2 generation all the diabetics were homozygous RT1^{u/u} (331). Continued breeding studies produced diabetics which were RT1^{u/l} or RT1^{u/b}, but no diabetics carried the genotype RT1^{l/l} or RT1^{b/b} (331, 334). Thus, at least one

u haplotype is a necessary prerequisite for the disease expression.

There is no apparent difference between the homozygous and heterozygous u haplotype bearing diabetics in relation to the age of disease onset or sex related incidence of diabetes, although IDDM occurs more frequently in the homozygous $RT1^{u/u}$ animals (335). As in human IDDM where affected siblings usually have one, and often both, haplotypes in common with the proband, irrespective of the HLA determinants making up the haplotype, so within BB rat litters where MHC haplotypes are segregating, diabetes occurs in animals with the same genotype. Thus, if the first animal to develop IDDM is $RT1^{u/u}$, subsequent diabetic animals will also be $RT1^{u/u}$. If the first diabetic animal is $RT1^{u/x}$, subsequent diabetics will also be $RT1^{u/x}$ (336).

To determine what regions of the associated MHC contribute to disease susceptibility, breeding studies have been performed that place recombinant haplotypes bearing the associated u haplotype in defined regions of the MHC onto the BB rat genetic background (337, 338). Transmission of the recombinant haplotypes is tracked in the resulting diabetic progeny using Southern blot hybridization with locus specific MHC probes. The results indicate that the IDDM susceptibility gene(s) map to the right of $RT1A$ and to the left of $RT1C$ and are probably associated with the class II $RT1D$ region. However, the MHC genes determining disease susceptibility were not unique to the BB rat (338).

This is supported by the findings of Holowachuk and Greer (339), where nucleotide sequence analysis indicates that the beta chains of the class II $RT1B$ and $RT1D$ molecules of the u haplotype from BB DP, BB DR and WF rats are identical. This suggests that class II molecules from BB

DP rats are not variant or unique and that unaltered class II molecules of the u haplotype support the autoimmune response in the BB rat. Thus, these reports correspond with the findings of Todd et al (177) which showed that human IDDM was not due to mutant class II genes.

Since the incidence of IDDM in crosses between WF rats (RT1^u) and BB rats is only slightly greater than crosses between BB rats and non-RT1^u animals, it suggested the presence of an IDDM resistance gene within the MHC (340). However, in recent breeding studies by Ono et al (338), diabetic animals homozygous for the WF derived RT1^u haplotype were identified, suggesting that any putative IDDM resistance genes are located outside the MHC. Furthermore, they report that although immunoregulatory defects e.g. lymphopenia, characteristic of some BB rats, may enhance the incidence of IDDM they are not obligatory for the expression of diabetes (338).

Recently workers from McDevitt's group in Stanford have investigated whether or not the aspartic acid negativity, at position 57 of the class II beta chains conferring susceptibility in human IDDM (177), is applicable to the BB rat model of IDDM (341). cDNA clones encoding the class II MHC genes of BB DP and BB DR rats were isolated and sequenced. The nucleotide sequences of the first domains of the RT1B beta and RT1D beta molecules were found to be identical in both the diabetes prone and diabetes resistant rats.

Although the BB rat was found to have a serine residue at position 57, in both the RT1B beta and RT1D beta sequences and therefore appeared consistent with the human studies (177), both the BB DP and BB DR rats possess the same sequence. Furthermore, they found that the region around amino acid 57 in the first domain of

both the RT1B beta and RT1D beta chains of the non diabetic Lewis rat (RT1^l) are identical to those of the BB rat. Moreover, the Buf rat (RT1^b) has a serine at position 57 as well (342). Thus, the allelic form of amino acid 57 does not appear to be a disease susceptibility marker in the BB rat model of IDDM. However, the absence of differences between the BB DP and BB DR sublimes, in their class II beta chain sequences, does not exclude these genes from being involved in the pathogenesis of the disease. Indeed, since with human IDDM susceptibility to diabetes is multigenic (336), the difference between the BB rat sublimes may be due to genes outside the MHC.

To further define the BB rat's RT1-linked diabetogenic gene(s), restriction endonucleases and MHC probes have been used to detect RFLP's between the BB rat and control strains at the genomic level (343-346). Buse et al (343, 344), using the restriction endonucleases BamHI and EcoRI in conjunction with a mouse class II (I-A alpha) gene probe, identified 4 polymorphic chromosome types (Ia, Ib, IIa, IIb) in the BB DR rats, whereas all the BB DP rats were homozygous (IIa/IIa). However, Kastern et al (345) failed to detect any class II gene polymorphisms using a human DR-alpha gene probe. The possibility that this probe lacked sufficient homology with the analogous rat genes, was subsequently tested using mouse genomic probes for I-A alpha, I-A beta, I-E alpha and I-E beta, but again no polymorphic bands were detected between the BB DP and BB DR rats (346).

Polymorphisms have however been reported using mouse class I MHC gene probes (344-346). Specifically, a 2Kb BamHI fragment was found in all BB DR rats, but absent in BB DP rats (345). Furthermore, a 40bP cDNA probe, representing part of the second extracellular domain of a class I antigen, identified 2 fragments; 15Kb and 7Kb in

the BB DR rats but only the 15Kb fragment in the BB DP rats (346). It was suggested that the gene contained in the 7Kb fragment may be deleted in the BB DP animals.

Other polymorphisms, including those at the 5'-flanking region of the rat insulin I gene; but not the insulin II gene, have been reported although the insulin gene alleles do not associate with IDDM in the BB rat (347).

In conclusion, diabetes in the spontaneously diabetic BB rat is inherited as an autosomal recessive trait (348), where susceptibility is linked to at least one gene associated with the rat MHC, but disease penetrance is modified by non-MHC genes. The presence of at least one u haplotype appears to be a necessary prerequisite for disease development. Specifically, disease susceptibility appears to be linked to gene(s) that map to the class II RT1D locus. However, the MHC class II genes determining disease susceptibility are not unique to the BB rat and, therefore, unaltered class II molecules of the u haplotype can support the autoimmune response leading to IDDM in the BB rat.

HUMORAL IMMUNITY

Unlike human IDDM, antibodies to islet cell cytoplasmic antigens are not found in the serum of BB rats (349, 350). However, islet cell surface antibodies are present in the BB rat.

Islet cell surface antibodies were first reported by Dyrberg et al (351) using a ^{125}I -protein A radioligand assay with dispersed Wistar rat islet cells, in 12 of 14 diabetic BB rats studied 3-11 days after diagnosis of IDDM. Subsequently, using the rat insulinoma cell line RINm5F (352), they found ICSEA in the prediabetic period in 16 of 22 BB DP rats (353). Islet cell surface

antibodies were present in some animals at weaning and therefore present at an age when no morphological or metabolic disturbances of the diabetic condition were detected. In these studies (351, 353), the presence of ICSA also correlated with that of anti lymphocyte antibodies, although the time course of the two antibodies varied (353).

The frequency of ICSA in the prediabetic period, and their subsequent predictive value for impending IDDM, is a contentious issue. Pollard et al (354), using an indirect immunofluorescence assay on pancreatic islet cell suspensions from cultured rat islets, detected ICSA in all of 12 prediabetic and 11 diabetic BB rats. More recently, Pipeleers et al (355) reported ICSA in diabetic but not prediabetic BB rats. The occurrence of ICSA was related to the time of clinical onset and to the age of the animals, with ICSA more frequent in animals developing IDDM at an earlier age. Although ICSA can be detected prior to the onset of IDDM (353), in one study there was no correlation between the development of IDDM, glucose intolerance or insulinitis and the levels of ICSA (356).

Monoclonal islet cell surface antibodies have been produced by fusing diabetic BB rat splenocytes with rat myelomas (357-359). One such MoAb termed IC2 bound to the surface of 56% of normal rat islet cells and 72% of rat insulinoma cells (357). In subsequent double labelling experiments, with an anti-insulin antibody, only insulin positive cells bound the IC2 antibody thus showing its beta cell specificity (358). However, expression of the cell surface antigen depended on the functional state of the beta cells, since islet cells isolated from 24-hour fasted animals showed a marked reduction in the number of insulin positive cells that bound the IC2 antibody.

Recently another ICSA MoAb, termed EC52, was identified and was cytotoxic to RINm5F cells but not other non-insulinoma cell lines (359). The EC52 antigen is present in normal pancreatic islets but is hidden by sialic acid residues and can be exposed following neuraminidase treatment. Following neuraminidase treatment, EC52 was found to stain 75% of insulin positive cells, but none of the insulin negative cells. In addition, after, but not before, neuraminidase treatment of the islet cells, EC52 was capable of exerting complement dependent antibody mediated cytotoxicity towards the rat islet cells. Using immunostaining of glycolipids separated by thin layer chromatography, hapten inhibition assays with defined carbohydrates and Western blots, the antigens recognized by EC52 on RINm5F cells were identified as glycoproteins with molecular weights of 60K and 68K. The antibody recognizes a carbohydrate antigen containing the sequence Gal beta 1-4 Glc NAc-R which on RINm5F cells is predominantly hidden by covalently bound sialic acid. It was therefore suggested that hidden antigenic determinants on islet cells, if exposed, may then become the target of autoimmune attack (359).

Complement-fixing islet cell antibodies, mediating islet cell cytotoxicity, have been documented in the BB rat (360). Using dispersed rat islet cells, CF-ICA were detected in 13 of 14 BB rats prior to the onset of IDDM. In 4 animals CF-ICA were present 4-8 weeks prior to the onset of disease. In 13 of the rats CF-ICA persisted 2-3 weeks after the onset of IDDM.

A possible pathogenetic role for ICSA was supported by Laborie et al (361). Serial serum samples were isolated from BB rats in the prediabetic period. All sera from animals that subsequently developed IDDM displayed complement dependent antibody mediated cytotoxicity to ⁵¹Cr labelled islet cells. Toxicity was specific for

islet cells since hepatocytes and fibroblasts were unaffected. Maximal toxicity occurred either just before or immediately after disease onset. Sera from diabetic animals also inhibited insulin secretion from normal rat islets either in the presence or, in some cases, the absence of complement. Beta cell specificity was shown with glucagon and somatostatin functions unaffected (361).

Antibodies to the 64KD islet cell protein found in human IDDM (223, 224) are also present in the BB rat (362). The 64KD protein was detected in rat islet cell fractions, immunoprecipitated with sera from all diabetic BB rats and 11 of 12 prediabetic BB DP rats. Antibodies to the 64KD protein were also found in BB DR animals at a lower frequency, but were absent from WF rats. In some BB DP animals, studied prospectively, antibodies to the 64KD protein were detected up to 8 weeks before the onset of IDDM.

Detection of anti-64KD antibodies may precede the development of ICSEA (356), however this may only reflect a higher sensitivity of the immunoprecipitation technique. The 64KD rat antigen has the same electrophoretic mobility as the human counterpart detected in human islets with human sera (223). The autoantigens may be identical since some human sera precipitate the 64KD protein from normal rat islets (227).

Other autoantibodies similar to polyendocrine autoimmunity in humans, perhaps reflecting abnormal immune regulation with resulting polyclonal B cell activation, are found among BB rat strains. Antibodies to smooth muscle, thyroid colloid and gastric parietal cells (349, 350), along with anti-lymphocyte antibodies (351, 353), have been reported as part of a general

autoantibody background in the autoimmune BB rat. In older diabetic rats, parietal cell antibodies were associated with lymphocytic gastritis and squamous metaplasia of the gastric mucosa (349). Antibodies to adrenocortical cells or thyroid microsomal antigens, however, have not been found (349, 350).

Thus, several of the features of humoral autoimmunity found in human IDDM, are also common to the BB rat. However, as with its human counterpart, humoral autoimmunity is believed to be of secondary importance behind cell-mediated immunity in the pathogenesis of IDDM in the BB rat.

CELLULAR IMMUNITY

Histopathology

In a study by Seemayer et al (312), light microscopy, immunohistochemistry and electron microscopy were used to document the appearance of insulitis in diabetic and normoglycaemic BB DP rats. Three groups of diabetic rats were identified : early diabetes (1-3 days after detection of glycosuria); stable diabetes (41-63 days after detection) and unstable diabetes (7-22 days after detection).

In early diabetes, islets were extensively infiltrated with activated lymphocytes and macrophages, and beta cells showed marked degranulation, injury and necrosis. Glucagon (A) and somatostatin (D) cells were largely unaffected. In stable and unstable diabetes, islets were small and markedly depleted of beta cells, but A and D cells remained intact. Variable degrees of insulitis, beta cell degranulation and necrosis were found in 65 day old normoglycaemic BB DP rats, indicating that the process of islet destruction is initiated well in advance

of the onset of clinical symptoms. In addition, the pancreata from diabetic and the 65 day old BB DP rats also showed mononuclear cellular infiltrates, distinct from insulitis, at periductular and/or acinar locations.

Use of a pancreatic biopsy technique allowed Logothetopoulos et al (363) to correlate the appearance of insulitis with the subsequent development of IDDM. Islets in biopsies taken 18-53 days before the onset of IDDM, showed normal structure and no insulitis. Biopsies removed 2-9 days before onset of disease, displayed extensive insulitis. In biopsies which preceded disease onset by 11-16 days, a small number of islets showed small focal mononuclear cellular infiltrates. Thus, insulitis appeared to develop rapidly from 2-3 weeks prior to the onset of IDDM.

Cell-Mediated Immunity

Lymphocytes, with reactivity for pancreatic antigens, are present in the circulation of BB rats. T lymphocyte lines, specific for islet cell antigens, have been isolated from the spleen and pancreas of newly diabetic BB rats (364). The T cell lines appeared to be of the T_H phenotype and displayed MHC restricted proliferation and IL-2 secretion in the presence of islet cell antigens. Responsiveness to islet cell antigens was inhibited by a MoAb against the class II RT1D antigen, suggesting that MHC class II antigen restriction of islet cell recognition by T cells occurs (365).

Cell-mediated immune destruction of pancreatic islet beta cells, as assessed by decreased insulin content following culture of rat islet cells with splenic lymphoid cells, and lysis of ^{51}Cr -labelled RINm5F cells, was detected in diabetic and BB DP rats (366). Cytotoxicity was specific for islet cells since WF or Lew rat spleen cells or a rat

pituitary cell line were not lysed (367). Anti-islet cellular cytotoxicity was mediated against both MHC-compatible and-incompatible islet cells suggesting a non-MHC restricted killing mechanism. Natural killer cells were identified as the cytotoxic effector cell since incubation of spleen cells, with an antiserum to NK cells, decreased the cytotoxicity (367).

An important role for NK cells in the pathogenesis of IDDM in the BB rat has been supported by other studies where NK cell numbers and NK cell activity have been increased in BB DP rats (368) and administration of antibodies against NK cells in vivo can prevent disease onset (369).

In an attempt to monitor the involvement of cell-mediated immunity in the target organ, autologous transfusions of ¹¹¹Indium-labelled peripheral blood lymphocytes were followed by gamma camera imaging and autoradiography (370). However, radioactivity recovered in the pancreata of BB DP, BB DR and WF rats were identical and labelled lymphocytes did not image the pancreas in relation to the presence of insulinitis.

In another study, recombinant IL-2 was radio-labelled with iodine and injected in vivo into BB DP rats (371). Combined immunoperoxidase staining and autoradiography of organ sections revealed that IL-2 bound specifically in vivo to IL-2R positive cells in the spleen of both normal and BB DP rats and to activated lymphocytes infiltrating the pancreas of BB rats. The severity of insulinitis correlated with the degree of radioactivity in the pancreas, and time-activity curves, generated over organs of injected animals after gamma camera imaging, confirmed the radioactivity was greater in the pancreas of BB DP rats than normal WF rats.

Lymphopenia, Circulating Lymphocytes and Abnormal Immunoregulation

Many of the early studies in the BB rat identified a profound T cell lymphopenia affecting peripheral blood, spleen, lymph nodes and thymus (316), which was initially believed to be a necessary prerequisite for the development of IDDM (317). As already mentioned, it is now established that lymphopenia is present in many BB rats, but it is not obligatory for the development of diabetes (318, 319, 338). However, the presence of lymphopenia in some animals has made it difficult to dissociate defects in immunoregulation, that may be pertinent to the aetiology of IDDM, with those that merely result from the lymphopenia defect itself.

Lymphopenia affecting total lymphocyte numbers is present in affected animals from birth (372). The lymphopenia primarily affects the T cell population with B cell numbers and serum Igs levels reported as normal (373) or slightly increased (374). Controversy exists as to which subpopulation of T cells is primarily affected, with some groups finding T_H cells markedly affected (316, 373, 374), whilst others reported diminished T cyt/supp cell numbers (375-377).

Recently, Greiner et al (378) have reported that the T cell lymphopenia in BB DP rats is largely due to the absence of the RT6 peripheral T cell subset. The RT6 alloantigenic system consists of 2 known antigens, RT6.1 and RT6.2 and approximately 50% of CD4 and 70% of CD8 peripheral T cells express RT6 (379). Genetic complementation studies indicate that the BB DP rat contains an intact RT6 gene, but fails to express the RT6.1 alloantigen in the functional absence of an accessory factor provided by RT6⁺ cells (380).

An immunoregulatory role for the RT6 subset was suggested when BB DR rats, with normal RT6⁺ cells, were treated with an anti-RT6 MoAb from 30 days of age and diabetes resulted within 2-3 weeks in 50% of the animals, compared with none of the controls (381). If anti-RT6 antibody treatment was delayed until 60 days of age, diabetes did not occur, thereby suggesting that during a critical developmental period, a regulatory population of RT6⁺ cells may prevent diabetes in BB DR rats.

Recently however, the reported absence of RT6⁺ cells in BB DP rats has been questioned. Lang and Kastern (382) have reported that the RT6 subset is present in BB DP rats in normal proportions when it is calculated on the basis of the total number of lymphocytes in any one animal and suggest that the previously reported decreased numbers of RT6 cells (378) results from, rather than causes the lymphopenia.

Studies aimed at identifying the origin of the T cell dysfunction, have been performed. Whereas Elder and MacLaren (373) reported a post-thymic defect, Scott et al (383) suggest two defects in the BB rat lead to the development of diabetes and/or lymphopenia. One defect occurs at the bone marrow stem cell, the other resides in the T cell differentiation environment.

Experiments using bone marrow transplants in both BB DP and BB DR rats, to create reciprocal histocompatible chimaeras, showed that disease susceptibility and resistance resided in the donor derived bone marrow and not the host genetic background (384). Georgiou et al (385) suggest that bone marrow derived cells, that are not T cell precursors (probably dendritic cells or macrophages), influence the maturation environment in the thymus of otherwise normal BB DP T cell precursors. This is supported by van Rees et al (377), where it was noted

that from birth there was a marked reduction in the macrophage subpopulations in the BB thymic cortex, but not in spleen or lymph nodes. An intrathymic maturational defect of CD8 cells was reported, since they failed to increase in numbers shortly after birth.

Numerous abnormalities of immunodeficiency in cell-mediated immune responses have been found in diabetic and DP BB rats. Impaired ability to reject allografts (373, 375) or respond in mixed lymphocyte cultures (386), along with decreased responsiveness to T cell mitogens (373, 387), have been reported. This anergy is not restored by the exogenous addition of IL-2 (375, 386) and, a reduced ability of BB lymphocytes to respond to IL-2 has been suggested (373). The poor proliferative responses and low IL-2 levels are not due to an intrinsic T cell abnormality (387), but to suppression mediated by a population of macrophages (387, 388), since removal of macrophages from BB spleen cells removed the suppressive effect. Other studies (319, 373), however, have not found any inhibition of IL-2 levels.

T cells from newly diabetic BB rats failed to exert cytotoxicity to MHC compatible islet cells suggesting an absence of functional T cytotoxic cells (376). This is supported by the findings of Woda and Padden (389) who used a model of LCMV infection to study functional activity of T cyt cells.

Whereas 7 days after infection BB DR rats generated lymphocytes which were cytotoxic for LCMV infected syngeneic fibroblasts, BB DP rats failed to generate either functional or phenotypic T cyt cells. They suggest that in the absence of T cyt cells, NK cells may represent the major cytotoxic effector cell in BB DP rats.

The capacity of alveolar macrophages from diabetic BB rats to phagocytose and kill bacteria is also impaired (390). However, this appears related to the degree of insulin deficiency, since the defect improves following intensive insulin therapy and BB DP rats, remaining non-diabetic, show normal macrophage function.

The involvement of suppressor T cells in disease immunoregulation has also been suggested with decreased T suppressor cell numbers and deficient suppressor cell activity reported (375-377, 391). Bone marrow transplants, from normal donors to BB DP rats, can restore normal suppressor cell numbers and activity and prevent the development of diabetes (391).

In common with human IDDM, elevated levels of activated (Ia^+) T lymphocytes are present in the circulation of DP BB rats (392-394). The increased Ia^+ T cell population includes both T_H and T cyt/supp cells, but preferentially the T_H population (392). The increase in activated T cells appears to peak shortly before disease onset, but is independent of blood glucose levels (392).

After onset, levels usually return to normal within a week (393). Elevated levels of Ia^+ T cells (about 4%), detected before 50 days of age, accurately identified a prediabetic stage and predicted the future development of IDDM with 85% sensitivity (true positives) and 83% specificity (true negatives)(393, 394). The interval between detection of elevated activated T cell levels and the onset of diabetes ranged from 22-82 days (393), but detection of elevated levels preceded detection of insulinitis which only occurred just prior to the development of hyperglycaemia (394).

Islet Transplantation Studies

Studies have been performed to investigate whether failure of islet transplantation is due to recurrent autoimmunity in the graft or due to a classical allograft rejection process. To preclude allograft rejection, BB DP rats were rendered tolerant of WF rat histocompatibility antigens by inoculation of WF bone marrow at birth, and verified by the permanent acceptance of WF skin grafts (395). Survival of WF allografts were compared in tolerant BB DP rats, which had developed IDDM spontaneously, with tolerant non-diabetic littermates in whom diabetes was subsequently induced by the injection of streptozotocin (STZ). In contrast to the permanent survival of WF islets, in the tolerant STZ induced diabetics, there was a uniform recurrence of insulitis and hyperglycaemia in the tolerant BB DP recipients with spontaneous diabetes. Since allograft rejection was precluded by the induction of tolerance, recurrence of autoimmunity causing damage to the transplanted islets was indicated.

Islet allograft destruction, due to recurrent autoimmunity, has also been found in other studies (396-398). Transplantation of cultured islet cells and pituitary tissue (to control for the allograft response), from MHC-incompatible donors into diabetic BB rats, resulted in destruction of the grafted islets but not of the pituitary tissue (396). Thus, recurrent autoimmunity specific for pancreatic islet cells resulted in the destruction of the transplanted islets.

In a study by Weringer and Like (397), cultured pancreatic islets and adrenal cortex, from inbred rats of variable MHC, pretreated to reduce antigenicity (399), were transplanted into normoglycaemic BB DP rats. Islet grafts, from MHC-compatible and MHC-incompatible donors, evidenced lymphocytic insulitis in animals that became

diabetic or evidenced lymphocytic insulitis in their endogenous islets. Adrenal cortex tissue was unaffected indicating the anti-islet response was not a normal allograft reaction. In addition, the results suggest that immune assault on pancreatic islets is not MHC restricted. Furthermore, since islet transplants, from non-BB rat sources, were also destroyed, it indicates that the islet cell antigens in BB DP rats are not unique or abnormal.

The persistence of memory effector cells was indicated when islet grafts, from BB DR rats, were transplanted into diabetic BB rats of long standing duration (in whom insulitis was absent from their remaining endogenous islets), and the grafts revealed lymphocytic insulitis after transplantation (398).

However heightened autoimmunity in BB rats of recent onset, is indicated by the shorter graft survival of both isogeneic and allogeneic islets when transplanted into diabetic BB rats of recent onset (duration about 20 days) compared with established diabetics (duration over 10 weeks)(400, 401).

Allogeneic islets were less sensitive to the specific autoimmunity and survived longer than isogeneic grafts, in both recent onset and established diabetics. Postoperative administration of cyclosporine prolonged allograft survival in both diabetic groups, but only in established diabetics when isogeneic islets were transplanted. This suggests that immunosuppression, given at or around a peak period of autoaggression, is unable to protect the islets from destruction.

Passive Transfer Studies

Pertaining to Witebsky's postulates for the establishment of an autoimmune disease (22), perhaps the most important criterion is that transfer of the experimental disease to a normal recipient, by serum or lymphoid cells, should be possible.

Nakhooda et al (402), using lymphocytes from acutely diabetic BB rats and athymic nude mice as recipients, reported insulinitis in 58% of the recipient mice. However, Rossini et al (403), using lymphocytes from acutely diabetic BB rats when transferred into SWR/JM nu/nu athymic mice and nu/nu athymic rats, failed to produce either insulinitis, hyperglycaemia or glucose intolerance.

Since the transfer of experimental allergic encephalomyelitis (EAE) was reportedly enhanced by preculturing donor cells from affected animals with the T cell mitogen ConA (404), Koevary et al (405) tried this approach in the transfer of IDDM in the BB rat. Spleen cells, from acutely diabetic BB rats, were harvested and incubated with ConA for 72 hours. When given to young (30-40 days old) BB DP rats, insulinitis accompanied by hyperglycaemia was found in 60-90% of recipient animals within 2 weeks of transfer. Since fewer than 0.5% of BB rats spontaneously develop IDDM before 60 days of age, this indicated that diabetes had been successfully transferred. In this initial study, transfer to BB DR recipients was not successful. However, diabetes has subsequently been transferred to other strains of rats (406, 407).

Diabetes occurred in 15% of MHC-compatible WF rats about 12 days after transfer with ConA activated spleen cells from acutely diabetic BB rats (406). However, transfer was only possible following immunosuppression of

recipients with cyclophosphamide 24-48 hours before transfer. Immunosuppression with cyclophosphamide, neonatal thymectomy, splenectomy or rabbit anti-rat lymphocyte serum, was again necessary for the adoptive transfer to BB DR rats or F1 hybrid offspring produced by diabetic BB X Lew (RT1^l), BB X Brown Norway (RT1ⁿ), BB X Yashida (RT1^u) and BB X NEDH (RT1^g) matings (407).

The results indicate that alteration of the recipients immune system, via immunosuppression, permits the transfer of diabetes not only to BB DP animals but also to other rats that were either homozygous or heterozygous for the BB RT1^u haplotype. Additionally, they suggest that since antigenically normal beta cells were destroyed by the transferred effector cells, BB derived genes in the target beta cells are not obligatory. Irradiation of the ConA activated lymphocytes prevents the adoptive transfer of diabetes, indicating that proliferating spleen cells with the propensity for cytokine production may be necessary to enable disease transfer (408).

Recently, the transfer of diabetes to BB DP and BB DR neonates was reported (409). Intravenous infusions of ConA stimulated spleen cells, from acutely diabetic BB rats into 6-36 hour old neonates, induced insulinitis and diabetes. Interestingly, thymidine-labelled blast cells initially migrated and settled in various immature lymph nodes and in the spleen within 24 hours after injection. Labelled and unlabelled cells did not appear in the pancreatic islets until 5-8 days after injection. Thus, the target-specific blast cells appeared to undergo a short period of proliferation and maturation in the lymphoid organs before migrating to the pancreas.

Handler et al (410) investigated the possible role of soluble factors in the transfer of diabetes. Conditioned media was obtained from the incubation of spleen cells from acutely diabetic BB rats with ConA, and fractionated by molecular weight using Amicon filters. Conditioned media from the fractions above 50 Kd, injected into 30 day old BB DP recipients, successfully induced diabetes in 25% of recipients within 3-4 weeks after the first injection. Equally, conditioned media, using ConA activated spleen cells from BB DR, MHC-compatible WF and MHC-incompatible Buf rats, was able to induce diabetes in BB DP animals. Conditioned media also induced diabetes in BB DR rats but not WF rats. The results suggest a soluble factor obtained from normal splenocytes can activate an effector cell population present in BB DP and BB DR rats to cause IDDM.

Postulated candidates for this soluble factor include IL-1 (411), Tumour necrosis factor (TNF)(412) and Interferon (IFN) gamma (413), which are cytotoxic to islet beta cells in vitro. Equally, IL-2 has been implicated since treatment of BB rats with recombinant IL-2 doubled and accelerated the rate of diabetes in BB DP rats (414). However, another study indicates that IL-2 is not the diabetogenic mediator (415).

Prevention Studies

Further indications for the involvement of autoimmunity, in the pathogenesis of IDDM in the BB rat, are suggested by the many reports documenting disease prevention following manipulation of the immune system via immunosuppression or immunomodulation.

Reconstitution of the BB DP rat immune system, either by bone marrow transplants (395, 416) or blood and spleen cell transfusions (417-422), have prevented the

development of diabetes. Weekly transfusions of whole blood from BB DR to BB DP rats, administered from 30-120 days of age, reduced the incidence of diabetes from 39% to 0% and restored diminished ConA responsiveness to normal control levels (417).

Subfractionation of the whole blood components, identified the white blood cell fraction as the active component mediating disease prevention (418). Furthermore, blood treated to deplete the T lymphocyte population failed to protect against the disease (418). A T cell mediated protection mechanism was supported by bone marrow transplantation studies (416). Whereas injection of MHC-compatible WF rat bone marrow into neonatal BB DP rats prevents the development of IDDM (395), if the bone marrow is pretreated to remove mature T cells then no effect on the incidence of diabetes is observed (416). Subsequently, T cell subpopulation analysis identified that transfusions, enriched for helper/inducer T cells, were found to afford the greatest degree of protection (419).

Effectiveness of transfusion depends on the dose of cells and recipients age at the time of transfusion. Accordingly, a single transfusion of more than 50×10^6 spleen cells, from either BB DR or WF rats, has been shown to reduce the incidence of diabetes when given to BB DP rats aged 27-46 days, but not older than 61 days (420). This further suggests, the presence of a critical developmental period in the BB rat around 30-60 days of age, during which a regulatory population of T lymphocytes may prevent the onset of IDDM. Once this temporal window is passed however, the ability of the regulatory T cell to ameliorate the disease process diminishes.

This is supported by the finding that spontaneous diabetes was fully prevented in the highly diabetes-prone BB/hooded (BB/h) hybrid rats, when given injections of nonactivated or ConA activated spleen cells from normal WF rats during the first two postnatal weeks, but the same postnatal injections did not protect against the adoptive transfer of diabetes later in life using ConA activated spleen cells from acutely diabetic BB rats (421). Therefore a regulatory mechanism may prevent the activation of self reactive lymphocytes but is unable to affect lymphocytes which have already been activated.

Additionally, MHC-compatibility between donor and recipient, enabling the persistent survival of donor cells in the recipient, is also necessary for successful transfusion to provide the cellular mechanism capable of abrogating the onset of IDDM (422).

Immunosuppressive protocols, including neonatal thymectomy (423) and total lymphoid irradiation (424), significantly reduced the incidence of diabetes. The earliest report of immunosuppression, which successfully reversed hyperglycaemia in 36% of diabetic BB rats and prevented the occurrence of diabetes in BB DP animals, was the administration of injections of rabbit anti-rat lymphocyte serum (ALS)(425). Subsequently, combined therapy with ALS and glucocorticoids along with cyclosporine A (CSA) improved the protection afforded by ALS alone (426).

Following the initial report by Laupacis et al (427), whereby administration of CSA from 30-100 days of age completely prevented the onset of diabetes or insulinitis in all of 40 treated BB DP rats, the use of CSA in the prevention of IDDM in both the BB rat (428-430) and in human IDDM (431) has received considerable attention.

Recently, use of the streptococcal preparation OK-432, which is a nonimmunosuppressive biological response modifier(432), successfully decreased the frequency of diabetes from 28% to 7% in BB DP rats (433). Cytotoxicity of spleen cells, towards RINm5F cells from OK-432 treated animals, was suppressed. However, the treatment of BB rats with OK-432 showed no suppressive effects on the spleen cell numbers, ConA responsiveness, peripheral blood T cell populations or ICSA titres, suggesting OK-432 has important nonimmunosuppressive immunomodulatory properties.

Since many of the above mentioned treatments result in widespread, almost total, suppression of the immune system, they give no indication as to the identity of the cells responsible for causing the disease. A role for macrophages in the pathogenesis of IDDM was suggested by studies whereby administration of silica (which preferentially affects macrophage function), virtually completely prevented the development of diabetes (434, 435).

Use of MoAbs to eliminate specific populations of cells has helped identify the phenotypes of potential candidates responsible for mediating beta cell destruction.

In studies by Like et al (369), in vivo depletion of specific lymphocytes using MoAbs against rat T cells and NK cells, reduced the incidence of diabetes from 61% to 0% and 12% respectively. Further support for the role of NK cells in the pathogenesis of IDDM was indicated when anti-asialo-GM1 antibody, which although not exclusively, primarily, affects NK cells, was given to diabetic BB rats tolerized to WF rat islet allografts and prevented the recurrent autoimmune destruction of the graft (436). Prevention of IDDM in the BB rat has also been achieved

following administration of a MoAb against rat class II MHC molecules (437) and antibodies to the IL-2R (438), thereby affecting activated T cells.

Other studies, at first seemingly unrelated to the previously mentioned immunosuppressive therapies, have also altered the course of IDDM development in the BB rat. Prophylactic treatment with insulin, from 50-142 days of age, prevented the development of diabetes in 28 of 36 BB DP rats (439). Since exogenous insulin decreases the endogenous insulin synthesis and secretion, it was suggested that beta cells having a low rate of insulin synthesis may be less vulnerable to autoimmune attack, or may reduce the amount of antigen(s) on the beta cell surface to a level insufficient to propagate autoimmunity.

In a further study, inoculation of 30 day old BB DP rats with LCMV (Armstrong strain, clone 13) prevented the subsequent development of diabetes in 63% of recipients (305). Inoculation with LCMV clone 13 caused selective immunosuppression by infecting a small subset (1-2%) of the peripheral circulating lymphocytes.

Finally, the development of IDDM in the BB rat may be influenced by alterations in the rat diet (303). Elliot and Martin (440) first demonstrated that the incidence of diabetes in BB DP animals was reduced from 50% to 15%, when from weaning, the standard chow diet was replaced by a semi-synthetic diet in which L-amino acids were substituted for natural intact proteins. Whereas the presence of wheat and milk proteins appear diabetogenic, other proteins such as hydrolysed lactalbumin prevented the onset of disease, and vitamin-E appeared able to modify disease progression after its initiation (441). Precisely how these dietary changes may influence or alter the immune system is not known.

Non-Obese Diabetic Mouse

The non-obese diabetic (NOD) mouse was discovered in 1974 at the Shionogi Research Laboratories in Osaka, Japan, having been derived from a subline of outbred Jcl-ICR mice in which spontaneous cataracts developed (442). Overt diabetes, occurring around 14 weeks of age, is characterized by hyperglycaemia, glycosuria and hypoinsulinaemia reflecting a specific loss of beta cells (442). Insulinitis begins around 4-6 weeks of age and is common in virtually all mice, but diabetes, however, preferentially ensues in female mice (80%) compared to 10-20% of the males (442). An involvement for sex hormones in the disease aetiology is suggested by the increased incidence (70%) of diabetes in males following castration, whereas female castration decreases the incidence (20%)(443).

Electron microscopy, used to examine pancreatic beta cells from NOD mice ranging from 18 day old embryos to 2 day old neonates, identified Type C retrovirus-like particles in the cisternae of the rough endoplasmic reticulum of these cells (444). The particles were found in 1-10% of all the beta cells in every NOD mouse examined and, as such, may have some role in the vertical infection from mother to child. In addition, maintenance of islets from CBA/J mice (which constitutively express these intracisternal retroviral particles) for 48h in 16.5mM glucose-containing medium has been shown to effect a 5-fold increase in the retroviral protein synthesis (445).

Genetic analysis indicates that the manifestation of insulinitis is controlled by two recessive genes on independent chromosomes (446), one of which is linked to the mouse MHC (447). Non-obese diabetic mice have a unique class II MHC, being unable to express I-E molecules due to the absence of mRNA for the I-E alpha

chain. Involvement of the I-E alpha gene in the development of diabetes was indicated when expression of I-E molecules in NOD mice, using C57BL/6 transgenic mice, prevented the development of insulinitis (448).

Insulinitis progresses from 4-6 weeks after birth and is made up of lymphocytes, macrophages, plasma cells and some neutrophils (449). As the mice age, the proportion of intact islets decreases with an increase in the number of islets showing severe insulinitis. However, islets free from insulinitis coexist with those displaying intense insulinitis, indicating that the progression of insulinitis is not uniform. The lymphocytes infiltrating the islets are predominantly Thy-1.2⁺ T cells, most of which are Lyt-1⁺ (T_H) cells, although Lyt-2⁺ (T cyt/supp) cells and NK cells are also found (450). Approximately half of the T cells expressed Ia antigens indicating their activation and B lymphocytes tended to increase in number with disease progression. Lymphocytic infiltration also occurs in other organs including the submandibular glands, thyroid and adrenal glands and testes and ovaries (451), which in the case of the thyroid and submandibular glands is accompanied by autoantibodies against these tissues (452).

Antibodies to islet cell structures including ICSA (453), ICA (452, 454, 455), and IAA (455, 456) have been found in the sera of NOD mice. Islet cell surface antibodies appear in the prediabetic stage in 10-50% of mice at 6-12 weeks of age and reach maximal prevalence (70%) just prior to onset of overt diabetes.

In one study, ICA have been found only at a low frequency (452) whereas in another study, a high frequency (60-80%) was found (454). Insulin autoantibodies have been found in 41% of pre-diabetic and 46% diabetic NOD mice (456). Both ICA and IAA appear to occur before the detection of

insulinitis, but the prevalence rate for each marker, or their degree of concordance, was different from the expected rate of diabetes (455). However, no animal developed diabetes without prior appearance of both ICA and IAA (455).

Studies on aspects of cellular immunity in the NOD mouse, in common with similar studies in the BB rat and human IDDM, give results which are often discordant. Whereas Kataoka et al (457) reported a decrease in the absolute numbers and percentage of circulating T cells, Miyazaki et al (450) found that the percentage of T cells was increased. In the former study, T_H cells were normal, whereas in the latter both T_H and T cyt/supp cells were increased. Similarly, higher values for the absolute number and percentage of circulating B lymphocytes was reported in the former, but the latter study found a lower percentage of B lymphocytes in the spleen of NOD mice.

Antibody dependent cellular cytotoxic activity in overt diabetics, but not prediabetic NOD mice, is increased (458), yet complement-dependent antibody-mediated cytotoxicity (CAMC) is not increased and there was no correlation between ICSA levels and CAMC levels in NOD mice sera (459). Natural killer cell activity and the function of cytotoxic T cells are found to be decreased (457, 460). Spleen cells from NOD mice are found to generate low IL-2 production and cell proliferation in response to ConA (461, 462). Similar to studies in the BB rat, evidence suggests that this impairment of IL-2 production is due to suppression mediated by activated macrophages (462).

Splenocytes from diabetic NOD mice were found to inhibit insulin release from stimulated islets, and depletion studies using MoAbs, identified the $Lyt-2^+$ population as

being responsible for the inhibition (463). L3T4 (T_H) cells were, however, involved in the recurrent autoimmunity responsible for destruction of islet allografts (464). Non-MHC-restricted cell mediated cytotoxicity towards ^{51}Cr labelled BALB/c islet cells has been reported, but the effector cell population was not identified (460). A cloned CD4^+ T lymphocyte line (BDC-2.5), with specificity for islet cell antigens, has been derived from NOD mouse spleen cells (465).

In an in vivo transplantation system, in which islet grafts were made in the presence or absence of the BDC-2.5 T cells, it was found that incorporation of the T cell line into the graft site resulted in the complete destruction of the transplanted islets, but not similarly transplanted pituitary tissue (465).

The involvement of cell-mediated immunity in the pathogenesis of diabetes in the NOD mouse is substantiated by the ability to transfer the disease using splenocytes from diabetic NOD mice (466). Transfer was induced within 12-22 days in 95% of irradiated NOD mice above 6 weeks of age. Spleen cells from 7 week old non diabetic NOD mice were unable to transfer disease, however, spleen cells obtained from 15 week old prediabetic NOD mice were able to transfer diabetes, indicating that sufficient effector cells are present at this stage and that the donor need not be overtly diabetic at the time of transfer.

Administration of 2 injections of cyclophosphamide (150mg/kg 2 weeks apart) can induce diabetes in 50-70% of male NOD mice (467). Injection of nicotinamide (500mg/kg) can ameliorate the effects of cyclophosphamide induced diabetes (468) and prevent lymphocytic infiltration into the submandibular glands (469). Nicotinamide injected into prediabetic NOD mice, also

successfully inhibits insulinitis and diabetes and, when given at disease onset, improves the diabetic condition (470). Furthermore, nicotinamide inhibits the ADCC reaction of mononuclear cells isolated from NOD mice spleen cells (458). However, in another study, nicotinamide was only able to prevent islet allograft rejection when combined with the iron chelator, desferrioxamine (471).

Reconstitution of irradiated NOD mice with bone marrow from BALB/c-nu/nu mice prevents the development of insulinitis (472), as does neonatal thymectomy (473). Administration of CSA (25 mg/kg every 2 days) to 30 day old NOD mice decreased the frequency of diabetes and inhibited cyclophosphamide-induced diabetes (474).

Other methods of immunosuppression, which ameliorate the development of diabetes in the NOD mouse, include whole body irradiation (475), the administration of silica (476) and injection of MoAbs against T cells (475) including the Lyt2 (476) and the L3T4 (477) T cell subsets. Non-obese diabetic mice treated every week from 4-24 weeks of age with the immunomodulating agent OK-432, were protected from insulinitis and the development of diabetes in all treated animals (478). The administration of monosodium glutamate also substantially decreased the incidence of diabetes and severe insulinitis (479). Monosodium glutamate may exert immunomodulatory effects through elevation of corticosteroid levels following monosodium glutamate-induced hypothalamic lesions.

Finally, comparable with the BB rat, the manifestation of diabetes may be abrogated by manipulation of protein-free diets (480).

LOW-DOSE STREPTOZOTOCIN DIABETES

Streptozotocin, a metabolite of the soil fungus *Streptomyces achromogenes*, was originally discovered in 1960 by Vavra and colleagues during a search for new antimicrobial agents (481). It was three years later, during an investigation of its antibiotic properties, that a diabetogenic effect was first reported (482). Thereafter, STZ was administered as a single high dose, causing complete beta cell necrosis and diabetes within 24 hours. In 1976 Like and Rossini demonstrated that diabetes could be induced in mice by 5 consecutive injections of subdiabetogenic doses (40 mg/kg) of STZ (483). Diabetes was not due to the direct toxic activity of the drug, and microscopy revealed mononuclear cells in and around the pancreatic islets. These observations led to the concept that administration of multiple subdiabetogenic doses of STZ, partially damages islets, thereby triggering an inflammatory response causing further beta cell loss and insulin deficiency.

Streptozotocin can damage the beta cell plasma membrane (484), or can act intracellularly where it is reported to deplete the islet content of NAD (485). Treatment of islets with nicotinamide prevents depletion of NAD and blocked the diabetogenic effect of STZ (486).

An important role for the generation of free radicals, in the diabetogenic action of STZ, is suggested by studies where the free radical scavenger superoxide dismutase (SOD) has been found to alleviate STZ-induced diabetes (487), and reduce the inhibitory effect of STZ on islet insulin release (488). Furthermore, combined treatment with the oxygen free radical scavengers, desferrioxamine and nicotinamide, prolong the graft survival of islet allografts in multiple low-dose STZ-induced diabetes (489). Streptozotocin can also induce strand breaks in islet cell DNA (490) and this leads to a cascade of

intracellular events including stimulation of DNA repair via poly-ADP-ribose synthetase (491), reduction of islet NAD content (492) and subsequent inhibition of islet function (493). The poly-ADP-ribose synthetase inhibitor, nicotinamide has been shown to protect the beta cell against these effects (494).

Outside the pancreas, STZ can also affect the immune system. Treatment with STZ has been found to reduce bone marrow, thymus and peripheral T cell numbers (495, 496), and suppressor T cell activity is reportedly enhanced (270, 497).

Similar to the NOD mouse, there is a strong sex influence on diabetes development. However, in the low-dose STZ model, it is the females who are largely resistant to the induction of diabetes (498); this can be overcome following castration or the administration of sex hormones (499). One or more genes within the mouse MHC influences the development of hyperglycaemia (500), but non-MHC genes also contribute to the control of diabetes susceptibility (501).

Spleen cell transfer experiments have been unable to induce persistent hyperglycaemia, although insulinitis did occur (502). Spleen cells with islet cell specificity displaying non-MHC restricted cytotoxicity against RINm5F cells have been reported (503), and ADCC activity is enhanced in low-dose STZ-treated animals (504). Following islet transplantation, reaction against the grafts only occurred if the recipients subsequently received a small dose of STZ, indicating that the grafted islets had to be stressed to permit an immune attack (505).

Although ICSA have been reported (506), the contribution of humoral immunity is assumed minimal since B lymphocyte depleted mice retain their susceptibility to STZ-induced diabetes (507). In common with the other models discussed and human IDDM, a multicellular insulinitis accompanies STZ-induced diabetes. Immunohistochemistry studies have identified T cells, including both T_H and T cyt/supp cells, NK cells, B lymphocytes and macrophages all contributing to the insulinitis composition (508).

Administration of silica particles almost completely prevents the development of diabetes, thereby suggesting a role for macrophages in the pathogenesis of low-dose STZ-induced diabetes (509). Equally, depletion of total T lymphocytes by the injection of a MoAb to Thyl, as well as depletion of both T_H (L3T4) and T cyt/supp (Lyt2) cells, protects against the development of hyperglycaemia and indicates the involvement of T cells in the generation of low-dose STZ-induced diabetes (508, 509). Administration of antibodies against MHC class II determinants (I-A) also prevents the onset of diabetes (510).

Other treatments reported to decrease the development of hyperglycaemia, include whole body U.V irradiation (511), administration of an interferon inducer, PICLC (512) and treatment with inosine pranobex, which increases IL-2 production (513). Suppression of serotonin-enhanced vascular permeability by methysergide or pargyline also reduced the incidence of diabetes (514).

Contrary to the findings in the BB rat and NOD mouse, administration of CSA enhances the development of low-dose STZ-induced diabetes (515, 516). Although CSA treatment may inhibit the development of insulinitis, diabetes probably results from the combined toxicity of CSA and STZ on the beta cells.

CONCLUSIONS AND AIMS

Since human IDDM is clearly a heterogenous disorder, no animal model can fully replicate all aspects of this multifactorial disease. However, the use of animal models have identified many common similarities as regards genetics, islet pathology and aspects of humoral and cellular immunity, between the models and the human condition. Ultimately, the use of animal models have stressed the importance of autoimmunity in the pathogenesis of IDDM.

The multiple low-dose STZ-induced diabetes model, is a useful tool to study how immune processes may augment the response to a beta cell cytotoxic agent. Nevertheless, any model used for investigating the true pathogenesis of beta cell destruction, must be one that spontaneously develops diabetes.

I have used the spontaneously diabetic BB rat as the model of choice for the experiments performed in this thesis, primarily because at the time of their conception, the NOD mouse was not available outside of Japan. However, since there is no sex difference on the propensity to develop diabetes in the BB rat, it probably better reflects the human disease. In addition, having the larger rodent enables certain investigations, that would prove difficult in the NOD mouse e.g. pancreatic biopsy, to be performed.

The aim of the studies detailed in this thesis was to better understand the aetiology of Type 1 diabetes. In particular : (i) to elucidate the precise sequence of events leading to mass destruction of pancreatic beta cells, by correlating changes in humoral and cellular immunity with pancreatic morphology in the prediabetic

period; (ii) in vivo and in vitro investigation of the role of aberrant expression of MHC molecules in the disease aetiology, and (iii) to examine the effects of immunosuppression on these events.

CHAPTER 4 - GENERAL MATERIALS AND METHODS



Fig 1: (A) non-diabetic DP BB/E rat (105 days of age);
(B) onset of diabetes in DP BB/E rat (105 days of age)

MATERIALS AND METHODS

ANIMALS

The Edinburgh colony of BB rats (designated BB/E) was established in 1982 from animals donated by Dr Pierre Thibert at the Animal Resources Division of Canada, Ottawa and is therefore derived from the original BB colony. The BB/E colony consists of two sublimes of animals created by selective breeding. The diabetes-prone mainline was established by selecting breeding pairs from high incidence litters, where at least 55% of the litter would develop diabetes. The incidence of diabetes in the DP BB/E line is 60-70% with a mean age at onset of diabetes of 96 days. The diabetes-resistant subline was derived by selecting rats from litters of low diabetic incidence (<30%). If any of the mating pairs subsequently developed diabetes at any stage of their lives, they, and all of their progeny, were disregarded. At the time of these studies, the incidence of diabetes amongst the DR BB/E rats was <2% at 120 days of age. At the time of writing, the incidence has been zero for the last three generations.

BB/E rats are maintained in rooms with independent heating and ventilation from the rest of the Animal Unit. Ventilation involves two sets of filters to produce 100% fresh air which is kept heated to $21 \pm 2^{\circ}\text{C}$, with the humidity at 50%. Animals are kept in an automatic light cycle of 12 hrs light/dark. Animals are allowed free access to food (No. 3 Expanded Breeding Diet, SDS, UK) and water. All animals are weighed twice weekly to monitor for the development of diabetes and give an indication of diabetic control in animals receiving insulin.

Detection and Management of Diabetes

Diagnosis of diabetes is based on a steady decrease in body weight (Figs. 1a & 1b), glycosuria and hyperglycaemia. Glycosuria is detected using Multistix (Ames Division, Miles Laboratories Ltd, Slough, UK) and results of 14mmol/l determined as positive. Blood glucose is measured following tail vein sampling, using BM-blood glucose test strips (BM-Test-1-44)(Boehringer Corporation (London) Ltd, Lewes, UK) in conjunction with a Reflolux-II blood glucose meter. Readings of >14mmol/l are determined as positive.

Following diagnosis, animals are maintained on one daily subcutaneous injection of Ultratard U-40 insulin (Novo, Copenhagen, Denmark) administered as near to 0900 hr as possible.

Other Animals

Normal Wistar rats, bred and maintained at the Animal Unit of the Western General Hospital, were obtained as controls when required.

Insulin Assay

Rat plasma insulin levels or islet insulin secretion following in vitro perifusion were measured by radio-immunoassay.

0.1mg freeze dried rat insulin (Novo, Copenhagen) was reconstituted with 1ml distilled water and used to prepare an insulin standard curve. 10 insulin standards (5-100uU/ml = 0.25-5ng/ml) were prepared following dilution in buffer B (6.2mg/ml $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; 0.24mg/ml thiomersalate; 5mg/ml BSA-Miles Fraction V; 9mg/ml NaCl and 25µl/ml trasylol, pH 7.4). 0.1ml of insulin

standards prepared in triplicate and test samples prepared in duplicate, were pipetted into LP3 tubes to which 0.2ml of anti-insulin serum (Wellcome Diagnostics, Dartford, UK) diluted 1:30,000 in buffer A (6.2mg/ml $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; 0.24mg/ml thiomersalate; 5mg/ml BSA) was added. Tubes were whirlmixed and incubated for 2h at 4°C. After incubation, 0.1ml (0.125 $\mu\text{Ci/ml}$) of ^{125}I labelled insulin (Amersham, Aylesbury, UK) was added to the tubes and further incubated at 4°C for 2h. Following the second incubation, 0.4ml of charcoal reagent (50mg/ml Norit GSX charcoal (Sigma, Poole, Dorset, UK) made up in heat-inactivated horse serum (Wellcome Diagnostics) diluted 1:2 with buffer A) was added and allowed to stand for at least 10 min. at 4°C. Following centrifugation (1500g for 25 min) at 4°C, 0.4ml of supernatant was removed into duplicate LP3 tubes using an LKB 2075 diluter (BCL, Lewes, UK) with 0.2ml distilled water washings between sampling. Tubes were counted using the NE 1600 gamma scintillation counter (Nuclear Enterprises, Edinburgh, Scotland) for 60 secs. Counts were expressed as a percentage of the total counts (0.1ml sample of ^{125}I labelled insulin) and a standard curve created with percentage total counts against units of insulin (mU/l).

Culture and Harvest of RINm5F Cells

The rat insulinoma cell line, RINm5F (352) was kindly supplied by Dr Anne Cooke (Department of Immunology, University College and Middlesex School of Medicine, London). RINm5F cells were continuously cultured at 37°C + 5% CO_2 in 50ml Falcon tissue culture flasks (A & J Beveridge Ltd, Edinburgh, Scotland) containing 5ml of tissue culture medium (RPMI 1640 containing glucose (11.1mmol/l), HEPES (20mmol/l), antibiotics (penicillin 100U/ml; streptomycin 0.1mg/ml) and heat inactivated 10% foetal bovine serum (Imperial Laboratories (Europe) Ltd, Andover, Hants, UK) until confluent. At confluence,

cells were harvested and split to allow continued growth of the cell line 2.5% trypsin (1:250) in Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} , Mg^{2+} (Imperial Laboratories) was diluted 1:10 in HBSS - Ca^{2+} , Mg^{2+} free at 37°C and 3ml added to each confluent flask to remove the adherent RINm5F cells, which occurred following 5 mins. incubation at 37°C . The free-floating RINm5F cells were transferred into sterile 15ml Falcon tubes (A & J Beveridge Ltd) and washed in 5ml tissue culture medium by centrifugation at 100g for 5 mins. Supernatants were poured off; the cells resuspended in tissue culture medium, split 1:8 and reseeded into fresh sterile tissue culture flasks topped with 5ml tissue culture medium. Media was changed every 2 days.

Isolation and Culture of Islets

Animals were killed by cervical dislocation and an immediate laparotomy performed to expose the pancreas and pancreatic duct. The distal duct was clamped off at its point of attachment to the duodenum. The proximal duct was cannulated with a Portex cannula (diameter 3FG)(Portex Ltd, Hythe, Kent, UK). Collagenase - type XI (1mg/ml)(Sigma, Poole, Dorset, UK) was made up in HBSS with Ca^{2+} , Mg^{2+} (Imperial Laboratories, Andover, Hants, UK) supplemented with penicillin (100U/ml) and streptomycin (0.1mg/ml)(Imperial Laboratories) and kept at 4°C . The pancreas was inflated by injecting 6ml of the collagenase solution over a 30 sec. period into each cannulated animal. The pancreata were carefully dissected from the attached gut tissue and placed on ice in a sterile 20ml container. Pancreata were incubated for 15-20 mins. in a water bath at 37°C and shaken at 60 cycles/min. The reaction was stopped by pouring the digest into 20ml cold HBSS supplemented as above and vigorously shaken for 30 secs.

Digests were centrifuged for a few seconds at 100g and supernatants poured off. Following a wash in HBSS with supplements, and removal of the supernatants, the digests were passaged X8 in a syringe with a 14 gauge steel needle in order to further disrupt the tissue. The digest was then passed through a sterile 500µm pore size nylon mesh (Henry Simon Ltd, Stockport, UK) and the filtrate centrifuged as previously. After decanting the supernatants, the digests were layered onto a Dextran gradient.

Dextran T70 (Pharmacia, Uppsala, Sweden) was made up in HBSS containing supplements to create a gradient with the following specific gravities: 1070, 1095, 1105 and 1125. Gradients were centrifuged at 4°C for 20 mins. at 450g. The first and second interfaces were removed and washed once in 50ml HBSS, followed by a further wash in 15ml HBSS. Islets were further purified from contaminating exocrine tissue by hand picking under a stereomicroscope.

Separated islets were washed X2 in 15ml HBSS and once in tissue culture medium, RPMI 1640 (Imperial Laboratories) containing glucose (11.1mmol/l), HEPES (20mmol/l), antibiotics (penicillin 100U/ml; streptomycin 0.1mg/ml) and heat-inactivated 10% foetal bovine serum (Imperial Laboratories). Islets were maintained in free-floating culture (37°C + 5% CO₂) in sterile petri dishes containing 5ml of RPMI tissue culture medium. Tissue culture media was changed 24h after islet isolation and thereafter every 2 days.

Determination of Islet Cell Surface Antibodies

Islet cell surface antibodies were estimated using a ¹²⁵I-protein A radioligand assay as described by Dyrberg et al (351). Dispersed islet cell suspensions were

prepared from beta-cell-rich foetal rat islets, or alternatively RINm5F cells were used as the islet antigen source (353). Plasma samples (10 μ l) were diluted 1:10 in RPMI 1640 (Imperial Laboratories) + BSA (5mg/ml)(Miles Laboratories Ltd, Slough, UK), and heat-inactivated (30 min. at 56°C). 0.1ml of islet cell suspensions or RINm5F cells (10⁶ cells/ml) were incubated with the heat-inactivated plasma samples for 60 min. at 4°C. After washing in RPMI + BSA (2 min. at 200g) the cells were incubated with 0.1ml ¹²⁵I-protein A (2x10⁶ cpm/ml)(Amersham International plc, Aylesbury, Bucks, UK) for 30 min. at 4°C. After 3 washes in RPMI + BSA and centrifugation at 200g, the radioactivity in the cell pellet was determined in a gamma scintillation counter (Nuclear, Enterprises, Edinburgh, Scotland). Cell bound IgG was expressed as cpm/10⁵ islet cells. Intra - and inter-assay variation was 6% and 14% respectively (N=8) with non-specific binding (islets incubated in the absence of plasma) contributing <0.02% of the total counts. Animals were regarded as being ICSA-positive when counts bound exceeded the mean binding values (\pm 3SD) for normal Wistar rats (518 \pm 159 cpm/10⁵ cells; N=32) and DR BB/E rats (486 \pm 138 cpm/10⁵ cells; N=32).

Detection of Insulin Autoantibodies

Insulin autoantibodies were measured by Dr Betty Dean (Department of Immunology, University College and Middlesex School of Medicine, London) using a micro-enzyme-linked immunosorbent assay (ELISA) modified from that described for the measurement of human IAA (243).

Purified rat, human or pig insulin (kindly donated by Novo, Copenhagen, Denmark) was dissolved in 0.05mol/l sodium carbonate-bicarbonate buffer, pH 9.6 and 0.1ml aliquots containing 100ng of insulin were pipetted into alternate rows of a 96-well flat bottomed microplate

(Nunc Immunoplate II, Gibco, Paisley, Scotland); the remaining rows (control wells) received 0.1ml of coating buffer alone. After 5h incubation the plates were emptied and washed (3x3 min., 220ul/well) with 0.015mol/l phosphate buffered saline (PBS) containing 0.1% Tween 20 (poly-oxyethylene sorbitan monolaurate)(Sigma, Poole, Dorset, UK). These and all subsequent incubations and washings were carried out at room temperature.

All wells were blocked for non-specific binding by incubation with PBS containing 20% normal rabbit serum (NRS)(Sera-Lab, Crawley Down, Sussex, UK) for 18h (120ul/well). The blocking solution was removed and rat plasma test samples (100ul diluted 1:50 in PBS containing 5% Tween 20 and 10% NRS) applied to the antigen-coated and control wells in triplicate and incubated for 2h. After removal and washing of the test samples, the plates were incubated for 1h with affinity purified rabbit anti-rat immunoglobulin horse radish peroxidase conjugate (Miles Scientific, Slough, UK), diluted 1:1000 in PBS containing 0.1% Tween 20 and 10% heat-inactivated NRS. The conjugate was removed and after 3 washes, 0.1ml of substrate was added to each well. Substrate solutions were prepared freshly by dissolving 3-3'-5-5'-tetramethyl benzidine (Aldrich, Gillingham, UK) in dimethyl sulphoxide (10mg/ml) in 0.05mol/l citric acid, 0.1mol/l phosphate buffer (pH 5.0, 100ml) and adding 20% Vol H₂O₂ (0.1ml) immediately prior to use. The reactions were terminated after 30 min. by addition of 12% H₂SO₄ (50ul/well) and plates were read at O.D. 450nm on an automated MR 580 microelisa reader (Dynatech, Santa Monica, CA, USA). For each assay, the instrument was set at zero using a well containing PBS - 0.1% Tween 20 (150ul).

The mean specific optical density at 450nm (Δ O.D.) was calculated by subtraction of the mean non-specific reading in the corresponding control wells. Reference plasma from normal Wistar and diabetic BB/E rats were included in each assay as controls. The intra - and inter-assay variation were 5.6% (N=5) and 15.2% (N=3) respectively using rat insulin and a standard diabetic rat plasma. The specific binding observed for plasma samples from normal Wistar rats (90-150 days of age; N=12) was Δ O.D. = 0.014 ± 0.018 , 0.071 ± 0.015 and 0.070 ± 0.013 (mean \pm SD) for rat, human and pig insulin respectively. Readings for test samples were considered positive when Δ O.D. values were at least twice the corresponding non-specific binding value and greater than mean \pm 3SD of the normal Wistar rat group for the relevant species of insulin.

IMMUNOFLUORESCENCE STAINING

Antisera

Table I lists the range of mouse anti-rat monoclonal antibodies used in these studies. I am greatly indebted to Dr Don Mason (Sir William Dunn School of Pathology, Oxford) who over several years has generously supplied me with clone supernatants of many of the MoAbs listed in Table I (OX-6, OX-8, OX-12, OX-17, OX-18, OX-19 and W3/25). The additional MoAbs, listed in Table I, were obtained as ascites fluid from Serotec, Oxford, UK.

Antisera to islet endocrine hormones were also used. Monoclonals HUI018 (anti-human insulin); GLU001 (anti-glucagon) and SOM018 (anti-somatostatin) and polyclonal antisera, M1183 & M1187 (guinea pig antibodies against insulin) were kindly donated by Novo, Copenhagen, Denmark. Polyclonal antisera against insulin (guinea pig antibody against beef/pork insulin) and glucagon (rabbit

anti-glucagon) were obtained (Incstar Ltd, Atlantic Antibodies, Winnersh, Berks, UK).

Tissue Preparation

Tissue on removal was immediately snap-frozen in isopentane at -70°C . Isopentane in a pyrex beaker was placed into a metal kidney dish containing acetone. Dry ice pellets were added to the acetone to cool the mixture, and the tissue dropped into the isopentane and allowed to stand for 1 min. All frozen tissue was stored at -70°C until required.

Tissues were mounted in O.C.T mounting medium (Raymond Lamb, London, UK) and serial sections (4 μm) were cut on a cold microtome cryostat (Slee Medical Equipment Ltd, London, UK) and sections placed on PTFE specialised coated multispot microscope slides (C.A. Hendley Ltd, Loughton, Essex, UK). Slides were air dried for 1hr and stored at -70°C .

Immunofluorescence

Cryostat sections, on removal from storage, were air dried under a cool air fan for 1hr, acetone fixed for 10 mins. at room temperature and screened with antisera. Clone supernatants were used at 1:2 - 1:10 dilution in phosphate buffered saline (0.05mol/l, pH 7.6) containing 10% normal rat serum (Sera-Laboratories Ltd, Crawley Down, Sussex, UK). Ascites fluids and polyclonal antisera were applied at 1:50 - 1:100 dilution in PBS containing 10% normal rat serum for ascites preparations, or 10% nonimmune serum from the host species in which the antisera were raised for the polyclonal antisera. All antisera were individually titrated using normal Wistar rat pancreas and/or spleen to establish optimal dilutions.

Sections were incubated with the antisera for 60 min. at 25°C in a humid chamber staining tray (Ortho, High Wycombe, Bucks, UK). Reactions of MoAb binding were revealed with species-specific biotinylated sheep F(ab¹)₂ anti-mouse IgG (Amersham, Aylesburgh, UK) diluted 1:50 - 1:100 in PBS containing 10% normal sheep serum, incubated for 40 mins., followed by fluoresceinated or Texas red-conjugated streptavidin (Amersham) diluted 1:100 - 1:200 in PBS and incubated for 20 mins. Alternatively, reactions of MoAbs were revealed after a 30 min. incubation with either fluoresceinated or rhodaminated rabbit anti-mouse immunoglobulins (Dakopatts, Weybridge, Surrey, UK). Polyclonal antisera were identified with the corresponding fluoresceinated or rhodaminated antibodies to guinea pig or rabbit immunoglobulins (Cambridge Bio Science, Cambridge, UK) diluted 1:20 - 1:60 in PBS containing 10% nonimmune host derived serum and incubated for 30 mins. at 25°C.

A 10 min. washing period, incorporating 3 changes of PBS, followed each antibody application. After the final wash, slides were mounted using a glycerol-phosphate buffered solution with an anti-fading agent (Citifluor Ltd, London, UK). Sections were examined under a Leitz Orthoplan microscope (E. Leitz Instruments Ltd, London, UK) fitted with a fluorescence vertical illuminator and filter blocks for revealing fluorescein and rhodamine/Texas red fluorochromes. Photographs were taken on Ektachrome ASA 400 film with automatic exposure using an Orthomat-W automatic microscope camera (E. Leitz).

TABLE I: MONOCLONAL ANTIBODIES USED IN STUDIES

MoAB	Isotype	Antigen or cell type recognised	Reference
MRC OX-6	IgG 1	MHC Class II (RT1B) - monomorphic	[517]
MRC OX-8	IgG 1	CD8 - thymocytes, T cyt/supp, NK cells	[518, 519]
MRC OX-12	IgG 2a	B lymphocytes (kappa light chain)	[520]
MRC OX-17	IgG 1	MHC Class II (RT1D) - monomorphic	[521]
MRC OX-18	IgG 1	MHC Class I (RT1A) - monomorphic	[521]
MRC OX-19	IgG 1	CD5 - thymocytes, T cells (pan T)	[522]
MRC OX-33	IgG 1	Rat LCA present on B lymphocytes	[523]
MRC OX-35	IgG 2a	CD4 - thymocytes, T _H , MØ	[524]
W3/25	IgG 1	CD4 - thymocytes, T _H , MØ	[525, 526]
MRC OX-39	IgG 1	CD25 - Interleukin 2 receptor	[527]
MRC OX-52	IgG 2a	CD5 - thymocytes, T cells (pan T)	[528]
ED1	IgG 1	Monocytes, MØ, Dendritic cells	[529]
ED2	IgG 2a	Tissue macrophages	[529]

CHAPTER 5 - HUMORAL AUTOIMMUNITY IN THE PATHOGENESIS OF
IDDM

INTRODUCTION

As in man, IDDM in the BB rat develops against a background of heightened autoimmunity where multiple autoantibodies can be detected in the prediabetic period. Antibodies to islet cell surface antigens (351, 353), gastric parietal cells and smooth muscle cells (349, 350), have all been found. The precise relationship of these autoantibodies to the development of diabetes, however, is uncertain. One way to evaluate the possible role of humoral immunity in the pathogenesis of IDDM is to ascertain if the antibodies appear before the clinical onset of diabetes. A positive affirmation would suggest that the presence of specific autoantibodies is a long-term process, preceding the manifestation of the disease, whereas the appearance of humoral markers only at, or after the onset would suggest they are a consequence of the disease. Longitudinal prospective studies required to answer these questions are essentially impossible to perform in man. One value of a model such as the BB rat is its potential for performing longitudinal studies in which various parameters can be assessed for their predictive power.

The studies detailed in this chapter were designed to investigate the role of humoral autoimmunity in the pathogenesis of IDDM, by attempting to correlate changes in humoral immunity with changes in both pancreatic morphology (i.e. insulitis) and function (i.e. islet insulin secretion).

TABLE II: BB/E RATS USED IN LONGITUDINAL STUDY I

Normoglycaemic Study Rats (age in days)	Parental Phenotype			
	DP BB/E		DR BB/E	TOTAL
	DXND	DXD	NDXND	
30	8	4	4	16
45	8	4	4	16
60	8	4	4	16
75	8	4	4	16* ^a
90	9	3	4	16* ^b
105	13	1	2	16* ^c
TOTAL	54	20	22	96

Excluded from study: *^a 3 rats; *^b 1 rat; *^c 3 rats all DP BB/E (DxND mating). Diagnosed diabetic (plasma glucose >14mmol/l) on study day. D: diabetic. ND: non diabetic.

MATERIALS AND METHODS

Two types of longitudinal study were performed.

STUDY I

Animals

Details of the cohort of BB/E rats used in this study are shown in Table II. 96 normoglycaemic rats (74 DP BB/E, 22 DR BB/E) were studied in groups of 16 animals at 15 day intervals between 30-105 days of age. During the study 7 DP BB/E animals were subsequently diagnosed diabetic (mean plasma glucose \pm SEM; 19.8 ± 1.2) and excluded from the study (Table II).

Protocol

At each time point, animals were sacrificed under light halothane anaesthesia. Following cardiac puncture, 5ml of blood was removed and plasma separated for the determination of plasma glucose (glucose analyser - Beckmann Instruments Inc, Fullerton, CA), insulin concentration and autoantibodies. Plasma samples were stored at -20°C until required. The pancreata were rapidly excised and a portion snap-frozen for immunofluorescence studies (Chapter 6) as detailed in Chapter 4. A further portion of the pancreata was used to prepare isolated islets for insulin secretion studies in a multichannel perfusion system.

Autoantibodies

Islet cell surface antibodies and insulin autoantibodies were estimated as previously detailed in Chapter 4. Cytoplasmic islet cell antibodies, and antibodies to gastric parietal cells (GPCA), smooth muscle (SMA) and

thyroid colloid antigens (TCA) were investigated by indirect immunofluorescence.

Details of the immunofluorescence studies are given in Chapter 4. Briefly; plasma samples were applied undiluted to cryostat sections of normal Wistar rat pancreas, stomach or thyroid and incubated for 20 mins. at 25°C. Following washing in PBS, the sections were incubated with fluoresceinated goat anti-rat immunoglobulins (Nordic, Maidenhead, Berks, UK) diluted 1:20 - 1:40 in PBS, and incubated for 20 mins. After washing in PBS, the slides were mounted and examined under a fluorescence microscope. Positive and negative control sera, including incubation with the second layer conjugate alone, were included in all studies.

Islet Insulin Secretion

Insulin secretion from isolated islets was estimated following perfusion of islets in a multichannel system (530), by my colleague Dr Bone. Islets were isolated by collagenase digestion from pooled pancreatic tissue of ICSA-positive (N=16) and ICSA-negative (N=15) rats, sacrificed at 75, 90 and 105 days of age. On each experimental day, the islet insulin secretion was determined for up to 8 batches of 30 islets (4 channels each from the ICSA-positive and ICSA-negative rats). In the multichannel system, islets were equilibrated by a 40 min. perfusion with basal media (2.7mmol/l glucose) and then subjected to two consecutive stimulations with 16.7mmol/l glucose applied 20 min. apart. Samples of the perfusate were collected throughout and stored at -20°C until assayed for insulin (Chapter 4) by Mrs Myra Gilchrist. At the end of the second high glucose stimulation period, the islets were perfused for a further 20 mins. with basal medium containing no BSA and recovered from the perfusion chambers. The islets were

ultrasonically disrupted, and their protein content determined (Bio-Rad protein assay kit, Richmond CA). Insulin secretion was expressed as μ U IRI per μ g islet protein so as to correct for any possible differences in the size of islets isolated from ICSA-positive and ICSA-negative animals.

Insulitis

Detailed immunological examination of the pancreata are described in Chapter 6. However, insulitis was determined on pancreatic sections stained with Haematoxylin and Eosin in addition to immunofluorescence studies, using the MHC class II MoAb (OX-6)(Table I). A positive result was given when pancreatic sections showed infiltration covering $\frac{1}{3}$ - $\frac{1}{2}$ of 3 or more islets. Sections were viewed independently by two observers (Dr Betty Dean and myself).

STUDY II

Plasma samples obtained from the tail vein of 102 BB/E rats (61 DP BB/E, 41 DR BB/E) who had received 1-3 pancreatic biopsies between 30-150 days of age, were screened for ICSA and IAA as previously described. Pancreatic biopsies were performed by my colleague, Dr Adrian Bone, with the view to investigate beta cell replication (531). Although reference may be made to these studies, they will not be detailed in this thesis.

RESULTS

STUDY I

Plasma Glucose and Insulin Concentrations

Table III shows the plasma glucose and plasma insulin concentrations of the study animals. Plasma glucose concentration increased significantly in DP BB/E rats from 30 to 45 days of age and thereafter remained relatively constant at 8.3mmol/l. There was a 6-fold increase in the mean plasma insulin concentration between 30 and 90 days of age, which subsequently declined by 50% at 105 days of age. DR BB/E rats showed a similar pattern of results with a 5-fold increase in the plasma insulin levels between 30 and 75 days of age, and a significant increase in plasma glucose concentration between 30 and 45 days of age, which levelled out to a mean plasma glucose concentration of 8.2mmol/l from 60 days onwards. There were no significant differences in either plasma glucose or insulin values between age-matched DP BB/E and DR BB/E rats.

Incidence of Autoantibodies/Relationship with Insulinitis

Antibodies against islet cell cytoplasmic antigens (ICA) and thyroid colloid antigens were not detected in any of the study animals.

Table IV shows the incidence of IAA in the DP BB/E animals. A low incidence of IAA was observed from 30 days of age, with the peak incidence of activity occurring at 90 days, close to the mean age of onset of diabetes in the BB/E colony. Insulin autoantibodies were never observed in outbred Wistar rats of comparable age. Table IV also shows the species binding specificity of the insulin antibodies. Most of the rat IAA appeared to recognize shared epitopes on rat, human and pig insulins. Although human and pig insulin appeared more reactive

than rat insulin in this assay system, among the 11 IAA positive DP BB/E rats, six plasma samples bound preferentially to rat insulin, three to human and one to both pig and human insulins. As expected, a high incidence of insulin antibodies was seen in diabetic animals maintained on injections of heterologous insulin.

Table V shows the incidence of rat IAA along with ICSA, GPCA and SMA in relation to the presence of insulitis in the DP BB/E animals. Antibodies against gastric parietal cells and smooth muscle were detected in some animals at all ages, with the frequency of GPCA approximately twice that of SMA. No apparent trend towards increased frequency, as the mean age for onset of diabetes approached or correlation with the presence of insulitis, was detected.

Islet cell surface antibodies were not detected in DP BB/E rats prior to 60 days of age, but were demonstrated with increased frequency thereafter. Insulitis was also not detected prior to 60 days of age but increased in frequency thereafter. Table VI shows the relationship between ICSA and IAA and the development of insulitis in the DP BB/E rats. Although ICSA was found in animals without insulitis (11/51) and insulitis was present in some animals without ICSA (3/16), a strong correlation ($r=0.91$; $p<0.01$) was detected between the presence of ICSA and insulitis. Insulin autoantibodies showed no significant correlation ($r=0.38$; $p<0.25$) with the development of insulitis, and there was no correlation ($r=0.18$; $p<0.4$) between ICSA and IAA in individual DP BB/E study animals.

Islet Cell Surface Antibodies and Insulin Secretion

The numbers of age-matched ICSA-positive and negative rats, did not allow a full statistical evaluation of islet insulin release. Therefore, the insulin secretory response to a glucose challenge (16.7mmol/l) was compared between pooled batches of islets isolated from groups of ICSA-positive and ICSA-negative rats aged 75, 90 and 105 days. Table VII shows the findings from three experiments and indicates a diminished total secretion of insulin in islets from ICSA-positive rats compared with animals without ICSA, with first phase insulin release being particularly affected.

STUDY II

Islet Cell Surface Antibodies, Insulin Autoantibodies and the Development of Diabetes

Only 4/39 (10%) and 1/31 (3%) of the DP and DR BB/E rats, respectively, were positive for IAA before 60 days of age. Thereafter, IAA were found in 16/48 (33%) and 3/29 (10%) of DP and DR BB/E rats respectively.

Table VIII shows the incidence of IAA and ICSA in relation to the development of diabetes. Since no animal younger than 60 days of age was positive for ICSA, only data from rats aged 60-150 days have been included for analysis. In this group (N=77; 48 DP BB/E; 29 DR BB/E), 28 (58%) of the DP animals and 4 (14%) of the DR rats subsequently developed IDDM. Among the DP rats which became diabetic, 20 had ICSA, 11 had IAA and 7 were positive for both antibodies. By contrast, in the group of 20 DP BB/E animals remaining non-diabetic, 4 had ICSA, 4 were IAA-positive and one animal was positive for both. In the DR BB/E rats, all four animals which developed IDDM were ICSA-positive, IAA-negative, but equally among

the 25 animals remaining non diabetic, 4 had ICSA and a further three rats had IAA.

A strong association of ICSA with the development of diabetes was observed ($\chi^2=8.3$; $p<0.005$). Insulin autoantibodies were less significantly associated with IDDM ($p<0.03$), using Mann-Witney U-rank test analysis of values from all Study II rats which developed diabetes (N=32) versus those remaining non-diabetic (N=45), and this low level of significance was confirmed ($p<0.05$) if DP BB/E rats alone were considered in the analysis. Similar analysis of the data from DP BB/E rats, revealed no significant association ($p<0.4$) of IAA with ICSA-positive (N=25) versus ICSA-negative animals (N=23).

TABLE III: PLASMA GLUCOSE AND INSULIN CONCENTRATIONS IN
PREDIABETIC BB/E RATS

DP BB/E RATS (age in days)	PLASMA GLUCOSE (mmol/l)	PLASMA INSULIN (mU/l)
30 (N=12)	7.1 \pm 0.2 [6.6 \pm 0.1]	5.0 \pm 1.1 [4.4 \pm 1.0]
	p<0.0005	p<0.005
45 (N=12)	8.4 \pm 0.2 [7.7 \pm 0.2]	10.5 \pm 1.6 [5.6 \pm 0.3]
		p<0.0005
60 (N=12)	8.7 \pm 0.1 [8.8 \pm 0.4]	22.7 \pm 2.1 [18.6 \pm 4.1]
75 (N=9)	8.0 \pm 0.2 [7.9 \pm 0.3]	26.2 \pm 5.1 [27.2 \pm 5.5]
90 (N=11)	8.1 \pm 0.2 [7.9 \pm 0.2]	30.3 \pm 4.1 [18.5 \pm 3.8]
		p<0.001
105 (N=11)	8.5 \pm 0.4 [8.3 \pm 1.3]	15.3 \pm 2.1 [23.5 \pm 8.9]

Results are expressed as mean values \pm SEM. Numbers in parentheses are results from age-matched DR BB/E rats (N=4 for 30-90 day periods; N=2 at 105 days). P values indicate the significant differences between DP BB/E mean values for the groups of animals shown (Students two-tailed t-test).

TABLE IV: INCIDENCE OF IAA (STUDY I)

DP BB/E RATS (age in days)	INSULIN		
	RAT (% positive	HUMAN per group)	PIG
30 (N=12)	17	17	8
45 (N=12)	0	0	0
60 (N=12)	17	17	8
75 (N=9)	22	22	33
90 (N=11)	36	64	64
105 (N=11)	9	18	9
Insulin treated diabetic BB/E Rats (90-150 days) (N=32)	56	88	91

TABLE V: INCIDENCE OF AUTOANTIBODIES AND INSULITIS (STUDY I)

DP BB/E RATS (age in days)	INSULITIS	ICSA (% positive	IAA per group)	GPCA	SMA
30 (N=12)	0	0	17	58	33
45 (N=12)	0	0	0	50	50
60 (N=12)	8	33	17	75	33
75 (N=9)	22	22	22	89	33
90 (N=11)	54	45	36	54	27
105 (N=11)	64	82	9	82	36
Total DP BB/E (N=67)	24	30	16	67	36
Total DR BB/E (30-105 days) (N=22)	4	32	27	36	9

TABLE VI: RELATIONSHIP BETWEEN ICSA/IAA AND INSULITIS
(STUDY I)

Antibody Status	DP BB/E RATS	
	With Insulitis	Without Insulitis
	(N=16) (% positive per group)	(N=51)
ICSA ⁺ /IAA ⁻	56	21
ICSA ⁻ /IAA ⁺	6	16
ICSA ⁺ /IAA ⁺	0	4
ICSA ⁻ /IAA ⁻	19	67

TABLE VII: ICSA STATUS AND INSULIN SECRETION

ICSA STATUS	<u>INSULIN SECRETION</u>	
	FIRST PHASE INSULIN	TOTAL INSULIN
	(uU IRI/ μ g islet protein \pm SEM)	
ICSA positive (N=3)	98 \pm 14	414 \pm 43
	p<0.025	p<0.05
ICSA negative (N=3)	151 \pm 9	577 \pm 42

(P values: students two tailed t-test ICSA positive v ICSA negative)

TABLE VIII: INCIDENCE OF ICSA AND IAA IN RELATION
TO THE DEVELOPMENT OF IDDM (STUDY II)

	DP BB/E (N=48)		DR BB/E (N=29)	
	D (N=28)	ND (N=20)	D (N=4)	ND (N=25)
	(% antibody positive rats)			
ICSA	71	25	100	16
IAA	39	25	0	12
ICSA/IAA	25	5	0	0

DISCUSSION

These studies have assessed metabolic status, presence of circulating autoantibodies, islet cell function and pancreatic morphology in individual potentially diabetic animals, prior to the development of overt diabetes.

In these studies, ICA were not detected in any animal and thereby are in agreement with other published studies (349, 350). Since ICA are common in newly diagnosed IDDM patients (189), this remains a point of difference between the human and BB rat studies.

Antibodies against thyroid colloid cells were also absent from the sera of animals used in these studies. This differs from other studies which report the presence of TCA in the sera of prediabetic (349) and diabetic (350) BB rats. However, in the former study, animals from 2 different BB rat colonies were used, and marked differences were observed in the incidence of the various autoantibodies between the colonies. The failure to detect TCA in our studies may in part reflect differences between the various BB rat colonies.

Alternatively, it may reflect differences in methodology, since our studies used normal WF rat tissue as substrate, whereas Like et al (350) used tissue from non diabetic DR BB/W rats. In addition, much older animals (3-8 months) were used in the studies by Like et al (350). Furthermore, although we did not investigate the presence of lymphocytic thyroiditis in these animals, the presence of TCA does not correlate with thyroiditis since 50% of animals without thyroiditis have TCA (350).

Although we did not detect TCA, in a subsequent study, autoantibodies to thyroglobulin were found in approximately 30% of BB/E rats, the presence of which was

unrelated to MHC haplotype or the development of either diabetes or thyroiditis (532).

In these studies, antibodies against smooth muscle and gastric parietal cells were found in some animals at all ages but did not appear to correlate with the development of insulinitis. In these studies, GPCA were approximately twice the frequency of SMA. In a similar study by Elder et al (349), GPCA were more prevalent than SMA in one BB rat colony, but less frequent in another colony; once again showing colony related variations. We did not investigate any relationship between the presence of GPCA with gastric functional abnormalities e.g. achlorhydria, however, in other studies although GPCA was associated with histologic evidence of lymphocytic gastritis, no functional abnormalities were detected in any such animals followed for 9 months (349).

Insulin autoantibodies have been detected in the sera of newly diagnosed IDDM patients (235), but there is much debate as to their value as a predictive marker for impending IDDM. Whereas some studies have found no association of human IAA with the presence of ICA (237, 239), Dean et al (243), on examining the class distribution of IAA, reported that the presence of both IgG IAA and CF-ICA confer increased risk for future diabetes development than either marker alone. Although subclass analysis was not performed, our studies on the prevalence of IAA in the BB/E rat, do not indicate a significant role for IAA in the development of IDDM. Furthermore, within individual animals no significant correlation was found between the presence of IAA with ICSEA which proved to be a much better indicator for the development of insulinitis and diabetes.

The studies herein described, I believe, are the first to demonstrate the presence of IAA in the BB rat, but, as with GPCA and SMA, probably arise as a corollary of heightened genetic susceptibility in these animals rather than representing a prerequisite for the development of IDDM. How IAA, along with the other autoantibodies, develop in these animals is unknown. Tolerance to self antigens can be broken following the administration of cross-reactive antigens (92, 93) which provide new carrier determinants to which the recipient is not tolerant and thereby, can generate carrier specific T_H cells which can help autoreactive B cells, recognizing shared determinants on the cross-reacting antigen. Although tolerant to their own insulin, BB rats would not be expected to be tolerant to proinsulin, which is normally sequestered in the beta cell.

In the event of beta cell damage following insulinitis, insulin precursors may come into contact with the immune system and permit the development of IAA if cross reactivity with proinsulin existed. However, since IAA were detected in some animals from 30 days of age, at which time insulinitis with beta cell destruction has never been observed, this explanation seems unlikely. Since IgM class IAA have been demonstrated following common viral infections such as mumps, measles, chicken pox and rubella (533), it is possible that IAA arise as the development of anti-idiotypic antibodies to idiotype determinants on anti-viral antibodies that perhaps cross react with the insulin receptor or share antigenic determinants with insulin itself (127).

Related to the idiotype network (32), it has previously been shown that mice, immunized to ungulate insulins, develop in the primary response an idiotype antibody that is directed against the epitope of insulin that is recognized by the insulin receptor (124). These receptor

mimicking idiotypic antibodies, designated DM-Ids, were replaced spontaneously by anti-idiotypic antibodies (anti-DM-Id), that were insulin mimetic and recognized the insulin receptor as well as the DM-Id (539).

Using sera, obtained from animals of our BB/E colony, Dana Elias at the Weizmann Institute, Rehovot, Israel has demonstrated that BB/E rats developing IDDM, spontaneously produce anti-DM-Id; anti-idiotypic antibodies that also acted as anti-receptor antibodies (submitted for publication). BB rats that did not develop IDDM did not produce anti-DM-Id. However, IAA were present both in rats developing IDDM and those remaining non diabetic. In addition, 75% of those rats developing IDDM carried the DM-Id compared with 25% of rats that did not develop IDDM. Thus, a spontaneous DM idiotypic network may herald the onset of autoimmune diabetes mellitus, where the anti-DM-Id has a higher association with disease than the DM-Id.

The strong association of ICSA with both the development of insulinitis and diabetes, found in our studies, suggest a prominent role for these antibodies in the aetiology of IDDM in the BB rat. Although ICSA were first detected in the sera of diabetic animals (351), subsequent studies (353, 354), confirmed by our studies, report the presence of ICSA in the prediabetic period. In a recent study, the beta cell specificity of ICSA was reported (355) and hence relates to similar observations in human IDDM (219). However in the study of Pipeleers et al (355), ICSA were only detected at clinical onset of disease, and not during the prediabetic period. Furthermore, the detection of ICSA appeared to be age related. However, it is possible that their inability to detect ICSA in the prediabetic period, reflects a lower sensitivity of their indirect immunofluorescence assay compared to the protein-A radioligand assay used in our studies.

Equally, since they used purified islet beta cells, it is possible they are identifying a subset of ICSA which are only prevalent at high titre at onset of diabetes when most of the islet beta cells are lysed and beta cell specific antigens are in the circulation at high concentration.

In our studies, ICSA were not detected prior to 60 days of age, at which age insulitis was also first observed. Dyrberg et al (353), however, detected ICSA in 38% of animals aged approximately 50 days, but no details are given as to whether or not these animals simultaneously presented with insulitis. In our studies, ICSA were found in some animals without evidence of insulitis, thereby suggesting ICSA may precede the development of insulitis. However, since the whole pancreas was not examined for insulitis, it cannot be excluded that insulitis was not present in other areas of the pancreas not examined. Equally, insulitis was found in some animals without detectable ICSA. It is possible that titres of detectable ICSA may fluctuate similar to ICA in humans (202) and therefore were not detectable at the time of sampling. Therefore, these studies do not establish whether or not ICSA precedes the development of insulitis or vice versa. However they do indicate a strong correlation between ICSA and the development of diabetes and its pathological substrate insulitis, and therefore promote ICSA as a useful predictive marker for IDDM in the BB rat.

Although the numbers of age-matched ICSA-positive and negative rats did not allow a full statistical evaluation of islet insulin release, the results using pooled islets from ICSA-positive and negative animals, demonstrated a perturbed pattern of biphasic insulin release from ICSA-positive animals. This confirmed previous studies from our laboratory in which larger numbers of animals were

used and a positive correlation was found between the level of ICSA in individual BB rats and the amount of insulin released following glucose stimulation of islets isolated from the same animals (534).

In another study by Svenningsen et al (535), pancreas perfusion of 45-50 day old DP BB rats showed that, despite normal kinetics of insulin release, the total amount of insulin release during glucose stimulation was reduced compared with the DR BB rats. This was attributed to a lower pancreatic insulin content, and is supported by a recent report indicating a reduction in the beta cell volume in DP BB rats prior to the development of insulinitis (536). However, in our studies the results are expressed per unit islet protein and islets from ICSA-positive and negative rats have a similar relative insulin content (534).

The observed differences in the pattern of insulin release between the studies of Svenningsen et al (535) and the studies herein described, may be due to different experimental techniques (perfused pancreas against isolated islets) or by age differences of the study animals. Nevertheless, a progressive decline in first phase insulin release during an intra-venous glucose tolerance test was reported in prediabetic BB rats, 25-50 days prior to the onset of IDDM (537). However, a similar impairment of beta cell function was observed in some animals remaining normoglycaemic, thereby indicating that although DP BB rats with circulating ICSA and/or abnormal islet insulin secretory function often go on to develop IDDM, this is not always the case. Whereas ICSA may indicate that an autoimmune response against the islet beta cells has been initiated, other factors, e.g. the ability to increase islet cell replication (538), may determine the final outcome of disease severity.

The impairment of insulin release from ICSA-positive rats may suggest a pathogenic role for ICSA in the development of IDDM. Islet cell surface antibody-positive sera from human IDDM patients can mediate complement-dependent cytotoxicity of rat islet cells (213, 218, 219) and ADCC against human insulinoma cells (228). In the BB rat, sera from 13 of 14 diabetic BB rats showed complement-dependent cytotoxicity against rat islet cells, and in the majority, the cytotoxic ICSA appeared prior to the onset of IDDM (360). The presence of complement-dependent cytotoxic ICSA was confirmed in prediabetic and diabetic animals using a ^{51}Cr -release assay (361). In addition, ICSA-positive sera suppressed insulin but not glucagon or somatostatin release from isolated islets. Furthermore, although islet cytotoxicity was observed in the prediabetic period, maximal potency occurred just prior to, and immediately after, the onset of overt diabetes.

At present the nature of the autoantigen against which ICSA are directed, is unknown. In an attempt to identify autoantigens detected by BB rat sera, homogenates of normal rat islets were immunoprecipitated with BB rat serum (362). As a result, sera from 25 of 26 diabetic or DP BB rats reacted with a 64KD rat islet cell protein, which was not present on rat spleen lymphocytes. Measurement of ICSA revealed 22 of the 26 BB rats were also positive for ICSA. Whether the immunoprecipitating islet cell antibodies are the same as those identified in the protein-A radioligand assay, remains to be determined.

In summary, these studies have described the presence of a variety of autoantibodies in the circulation of prediabetic BB/E rats. The majority of these antibodies showed no pathologic sequelae and support the findings that autoantibodies that react with multiple organs are a

common feature of the normal immune repertoire (80, 81). Amongst the detectable autoantibodies, only ICSA showed a significant correlation with the presence of insulinitis and the development of diabetes. The results do not allow us to conclusively ascertain whether or not ICSA appear prior to beta cell damage and thereby perhaps initiate beta cell destruction, or whether they occur only after beta cell damage has been initiated. Nevertheless, the close association between ICSA and insulinitis, and ultimately diabetes, suggests an important role for ICSA in the pathogenesis of IDDM. Our findings of impaired insulin release in ICSA-positive rats, when taken together with other studies, indicate the potential of ICSA to participate in antibody-mediated beta cell destruction. However, it remains to be established whether ICSA play a primary or secondary role in the process of beta cell destruction.

**CHAPTER 6 - MHC EXPRESSION AND INSULITIS IN
THE BB/E RAT PANCREAS**

INTRODUCTION

The characteristic histopathology of Type 1 diabetes is "insulitis" (255), representing a mononuclear cellular infiltrate of the islets of Langerhans, leading to insulin deficiency as a result of selective beta cell destruction (194). It is not known what initiates this process or what cells of the immune response are responsible for mediating beta cell damage. However, an attractive hypothesis suggested that aberrant expression of MHC class II molecules on pancreatic beta cells may initiate an autoimmune reaction against the insulin containing cells (540).

The major histocompatibility complex antigens are cell surface glycoproteins, associated with the regulation of immune responses. Class I antigens are located on most nucleated cells and are responsible for restriction of T cyt cells (149). Class II antigens are required for presentation of antigen and are normally present only on the surface of antigen-presenting cells, activated T cells and B lymphocytes (148, 150). The observation that class II molecules were expressed on follicular cells in autoimmune thyroid disease (541), and that this expression could be induced on normal thyroid cells by the lectin phytohaemagglutinin (PHA)(542), led to the suggestion that aberrant expression of class II antigens may allow the presentation of specific autoantigens to potentially autoreactive T_H lymphocytes, thereby leading to autoimmune disease (540).

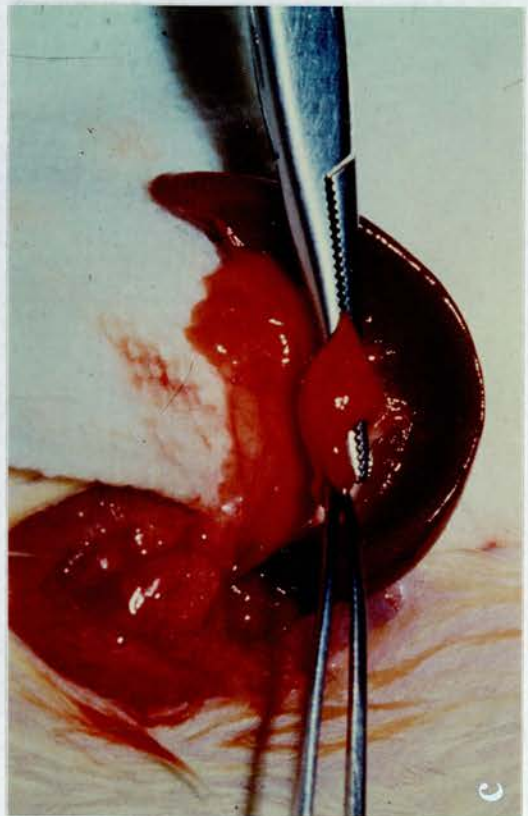
Such aberrant expression of MHC class II antigens on pancreatic beta cells has been described in human Type 1 diabetes (261). In this report of a single case with recent onset of Type 1 diabetes, examination of fresh frozen autopsy pancreas revealed two major abnormalities of MHC expression in some islets: firstly expression of class II MHC products specifically confined to islet

beta cells and secondly, marked hyperexpression of class I MHC molecules on all islet endocrine cells. However, since the patient presented at a time when the majority of the beta cells have been destroyed, the relevance of these findings to the events of the prediabetic pancreas are uncertain.

The studies detailed in this chapter aimed to elucidate the sequence of pancreatic events which lead to the development of IDDM in the BB/E rat. In particular, they sought to document the role of aberrant MHC expression in the disease aetiology and to perform phenotypic analysis of the infiltrating immunocytes.

Fig 2 : Pancreatic biopsy procedure :

- (a) incision along the linea alba
- (b) exteriorization of splenic pancreas
- (c) clamping of biopsy
- (d) removal of biopsy
- (e) application of thrombin to cut surface
- (f) release of clamp
- (g) suturing of wound
- (h) skin closure with wound clips





MATERIALS AND METHODS

Two longitudinal cohort studies were performed.

STUDY I

Pancreata examined in this study, were obtained from and correspond to the animals of Study I detailed in Chapter 5.

STUDY II

This study employed serial pancreatic biopsy, thereby allowing sequential study of pancreatic changes within individual animals. This study is not related to Study II described in the previous chapter.

Pancreatic Biopsy Procedure

In 1984, a technique for the complication-free removal of splenic pancreas from rats, was described by Logothetopoulos et al (363). I have visited Dr Logothetopoulos and observed this technique in his laboratory. Subsequently we have used this technique to study the development of IDDM in individual animals. Pancreatic biopsy was performed, essentially as described by Logothetopoulos, by my colleague Dr A J Bone with myself assisting.

Animals were anaesthetised with 2.5% halothane and a 2-3cm incision made along the linea alba from the umbilicus to the xiphisternum (Fig. 2a). The spleen with attached pancreas was gently lifted out and exteriorized on sterile gauze soaked in sterile saline. The splenic pancreas was carefully unfolded so that its vascular connections with the spleen were clearly visible (Fig. 2b). A portion (50-75mg) of pancreas from

just above the lateral splenic attachment was clamped off using a surgical haemostatic clamp, in which the arms were finely machined so that their thickness did not exceed 0.2-0.3mm (Fig. 2c). The biopsy was then removed using fine scissors (Fig. 2d). The cut surface was dabbed with sterile bovine thrombin (100U/ml)(Miles Scientific, Illinois, USA) without releasing the clamp (Fig. 2e). After 3-4 mins. the clamp was gently released (Fig. 2f), if bleeding occurred the clamp was left for a further 2 mins. The exposed spleen and pancreas were covered during this "clotting" period with sterile gauze soaked in sterile saline. The spleen and pancreas were then returned to the abdominal cavity which was closed using a tight running stitch with number 3/0 synthetic (coated vicryl) absorbable sutures (Ethicon Ltd, Edinburgh, Scotland)(Fig. 2g), followed by skin closure with wound clips (Fig. 2h). Although occasionally sutures were required to control haemorrhage, no difficulty was encountered in repeating pancreatic biopsy up to four times in individual animals.

Tissue obtained at biopsy was immediately snap-frozen for subsequent immunohistological examination.

Protocol

Eight groups (N=5) of DP BB/E rats were biopsied twice (with a 10 day interval) between 30 and 110 days of age and subsequently followed up to 200 days of age for the development of IDDM. Animals developing diabetes received a third biopsy at onset of disease. Subsequent maintenance on daily insulin was withheld from six animals to allow the formation of ketones. Following the detection of ketones, the animals were sacrificed and their remaining pancreata removed. Similarly, a group (N=6) of animals with established diabetes (mean

duration of diabetes; 23 ± 5 days), having been maintained on daily insulin injections, received a pancreatic biopsy prior to the cessation of their insulin treatment and received a second biopsy at the onset of ketoacidosis. Biopsies were also performed on DR BB/E animals (N=12) and on normal Wistar rats (N=6) between 100 and 120 days of age.

One hundred and twenty serial cryostat sections ($4\mu\text{m}$) were cut from each biopsy. Scan slides with 8 sections/slide, each section taken at a different level through $80\mu\text{m}$ of tissue, were used as an initial screen to detect areas of pancreatic infiltration with class II MHC positive cells using the MRC OX-6 MoAb (Table I). Detailed analysis of areas with infiltration, via the antisera listed in Table I, were performed by referring back to subsequent serial sections coincident with the areas represented by the scan slides.

Immunofluorescence Staining

Labelling of cryostat sections for immunohistological examination, by immunofluorescence procedures, were performed as detailed in Chapter 4.

Quantitation of Insulitis

Due to the heterogeneity of the insulitis process, quantitative analysis on both the total numbers and individual subset analysis of infiltrating cells, contributing to the insulitis is not practical. However, to give some degree of quantitation, I have studied a total of 46 islets from biopsies taken from 3

animals which subsequently developed diabetes 10-15 days later. At this stage, the insulinitis process is at its peak, yet heterogeneity in the degree of insulinitis within individual islets is observed.

Individual islets showing insulinitis were classified according to the number of MHC class II positive cells (OX-6) within the islet, in conjunction with the relative amount of insulin positive staining beta cells as follows:

++; 50-100 OX6⁺ cells; insulinitis located mainly at the islet periphery and the majority of the beta cells intact.

+++; 100-150 OX6⁺ cells, insulinitis affecting $\frac{1}{3}$ - $\frac{1}{2}$ of the islet with resultant loss of $\frac{1}{2}$ - $\frac{2}{3}$ of the insulin containing beta cells.

+++; more than 150 OX6⁺ cells; insulinitis covers most of the islet and there is a virtual absence of insulin staining cells (Fig. 3).

Two cell counts were performed on each islet area using X60 and X25 objective lenses, with the mean of the two scores reported. Except for the MoAb ED1, which recognizes a cytoplasmic vacuole antigen, cell surface staining was counted. Where difficulties existed in identifying the morphology of individual cells, the count was underscored.

Detection of Ketoacidosis

Ketones (3.9-15.7mmol/l) were detected using Multistix (Ames Division, Miles Laboratories Ltd, Slough, UK) and ketoacidosis confirmed by measurement of plasma carbon dioxide, sodium, potassium, glucose and creatinine levels on a Synchron AS4 (Beckman Instruments, Fullerton CA, USA). Typically raised potassium levels, due to

insulin-deficiency, causing increased release of intracellular potassium and lowered CO₂ levels, reflecting the formation of acidic ketone bodies, were found (Table IX).

TABLE IX: ELECTROLYTE AND OTHER BLOOD CONSTITUENTS
AT ONSET OF KETOACIDOSIS

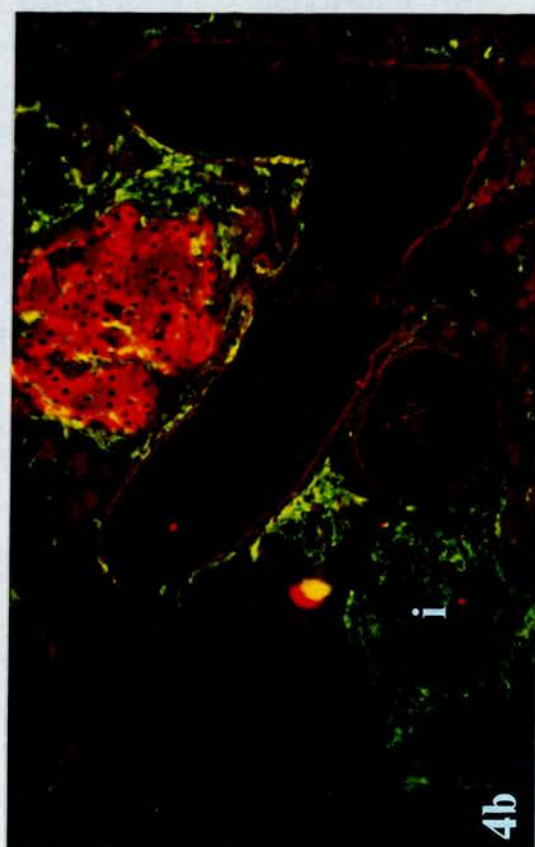
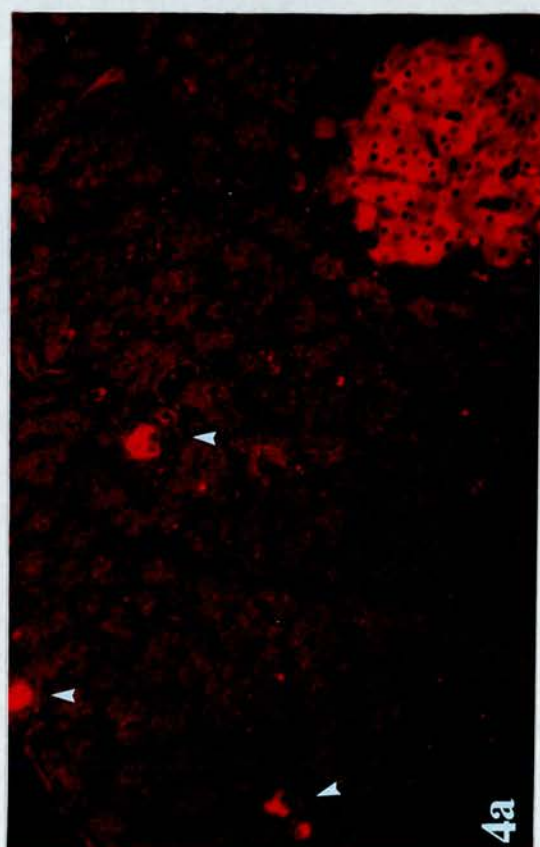
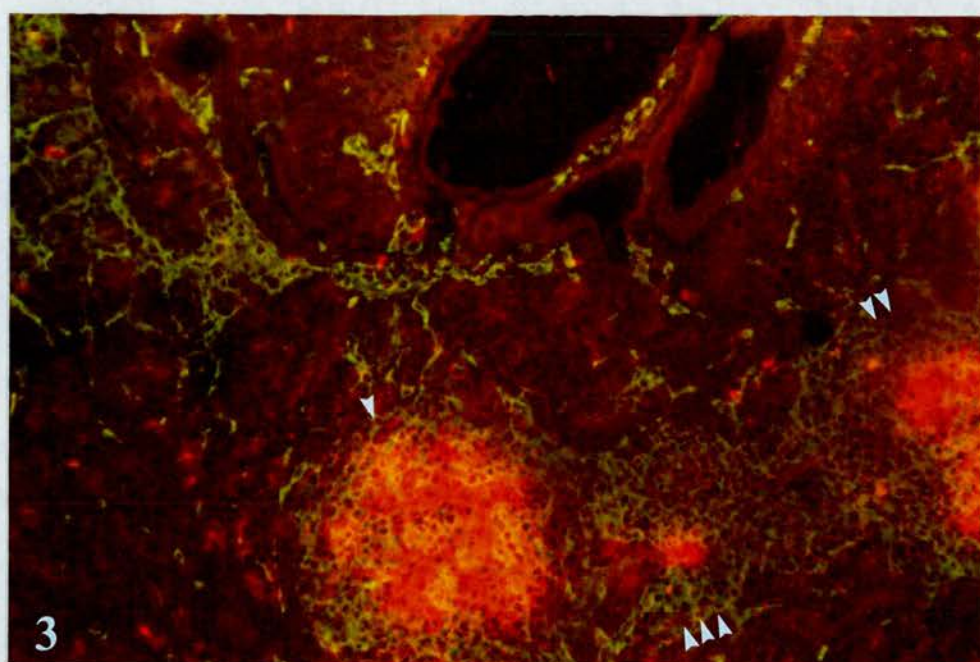
	CO ₂	Na	K (mean \pm SEM)	Glucose	Creatinine
Established diabetics (N=6)	22.4 \pm 1.9 (p<0.025)	144 \pm 5.0 NS	9.1 \pm 1.0 (p<0.001)	38.4 \pm 6.4 (p<0.0005)	71 \pm 11.4 (p<0.025)
Newly detected diabetics (N=6)	20.3 \pm 2.1 (p<0.025)	147 \pm 2.6 NS	6.4 \pm 0.7 (p<0.01)	36.5 \pm 5.2 (p<0.0005)	73 \pm 15.0 (p<0.05)
Non diabetic DR BB/E (N=6)	28.0 \pm 1.1	147 \pm 0.7	4.2 \pm 0.2	8.6 \pm 0.4	42 \pm 0.9

P values indicate the significant differences compared with the DR BB/E group (students two-tailed t-test).

Fig 3 : Double exposure of MHC class II positive (OX-6) cells (green) and insulin-containing cells (red) on pancreatic biopsy from DP BB/E rat (90 days of age). Biopsy was taken 11 days before onset of diabetes. Three islets with different levels of insulinitis are identified (arrows). (Magnification X 125).

Fig 4 : (a) Insulin staining (M1183) of DP BB/E rat pancreas (aged 90 days). Intact insulin-containing islet in one pancreatic lobule appears alongside three shrunken "end stage" islets with diminished insulin content (arrows). X 125

(b) Double exposure of OX-6 (green) and insulin (red) staining on DP BB/E rat pancreas (90 days). An insulin deficient islet (i) with insulinitis appears alongside another insulin-containing islet with early pre-islet infiltration. X 125



RESULTS

At the outset the heterogeneity of the pancreatic events in the prediabetic pancreas must be stressed. Therefore, each biopsy can only reflect a part of the total pancreas at a single time point. As such, these studies describe changes in pancreatic morphology leading to insulitis within individual and multiple islets. It is only with unrestricted spreading of this process, from one pancreatic lobule to the next, that eventually when sufficient beta cells have been destroyed that clinical overt diabetes ensues. Figure 4 shows the lobular distribution of pancreatic events typical of this "patchy" disease. In Figure 4a, the beginning and end of the destruction process are indicated, with three end stage shrunken islets with virtually no remaining insulin containing beta cells appearing alongside an intact insulin containing islet in an adjacent pancreatic lobule. In Figure 4b, the varied intensity and distribution of the insulitis process, responsible for beta cell destruction, is shown with the lower islet heavily infiltrated and no residual insulin-positive cells, whereas the islet in the lobule above the large blood vessel, shows only the early stages of insulitis.

STUDY I

Pancreata were screened with a MoAb against rat MHC class II (Ia) antigens to detect areas of infiltration and/or altered Ia expression on non lymphoid cells. Table X shows the percentages of pancreata revealing insulitis or abnormal Ia expression in the study animals. Figure 5 shows Ia expression in the normal non diabetic pancreas where 0-15 spheroidal or stellate cells of presumed monocytic origin may be found within islets or adjacent to capillaries scattered throughout

the exocrine tissue (543, 544). The first change was observed in the pancreata of 45 day old BB/E animals and consisted of increased expression of class II molecules on the vascular endothelium (Fig. 6). This preceded pancreatic lymphocytic infiltration and insulinitis which was first detected in one animal at 60 days of age. From 60 days onwards, 16 pancreata presented with various degrees of insulinitis which increased in frequency and severity as the mean age of onset of diabetes for the BB/E colony approached (Table X).

Analysis of the infiltrating cells revealed that in all cases, cells reactive with the W3/25 MoAb; indicative of T_H and macrophage phenotypes, were the predominant cell types representing approximately 60% of the infiltrating cells (Fig. 7a). Double fluorochrome studies indicated that the majority of these cells also expressed class II molecules suggesting their recent activation (Fig. 7b). OX-8 positive cells which can represent T cyt, T supp or NK cells identified approximately 15-20% of the infiltrates (Fig. 7c). Up to 90 days the presence of B lymphocytes amongst the infiltrating cells was approximately 10-15%, however in older animals approximately 30% of the infiltrate was composed of B lymphocytes, including plasma cells (Fig. 7d).

Interestingly, the T and B lymphocytes appeared to occupy distinct areas within the infiltrated islets.

Among 3 of the 16 pancreata with insulinitis (1 pancreas at 60 days; 2 pancreata at 75 days), occasional endocrine-like cells were observed to express class II molecules (Fig. 8). When sections were double stained for endocrine hormones, a few insulin containing cells appeared to be expressing class II antigens (Fig. 8c). The expression of MHC class II molecules on endocrine cells was specific for the insulin containing cells and

was never observed on the glucagon or somatostatin cells which remain intact and unaffected in this beta cell specific disease (Fig. 9). Aberrant class II expression on endocrine cells was however an infrequent phenomenon, being observed in only 3 pancreata, within which only a few of the islets with remaining insulin containing cells were affected. In all cases, this aberrant expression of MHC class II molecules was accompanied and coincident with lymphocytic infiltration of the islets.

STUDY II

The longitudinal cohort study described above, had several inherent limitations: (i) since the incidence of IDDM in the BB/E colony is about 70% it was not known which animals with insulinitis would have subsequently gone on to develop IDDM; (ii) it was not possible to assess the time scale of the insulinitis and beta cell destructive processes with any degree of accuracy; (iii) at the time the study was performed, the MoAbs available were unable to distinguish between the T_H subset and certain populations of macrophages. More recently however, several MoAbs identifying distinct rat macrophage subpopulations, have been described (Table I)(529). Using these antibodies in conjunction with serial pancreatic biopsy, which allows sequential study of individual animals over prolonged periods, this second study aimed to remedy the deficiencies of the previous study and refine its conclusions.

Although it is recognized that each biopsy only reflects the events within a portion of the pancreas, at a single time point, the performance of serial biopsy in individual animals at least allows the morphology of each biopsy to be interpreted as regards its relationship with the subsequent development of IDDM in the animal.

By pooling the observed changes in pancreatic morphology, within individual animals and relating them to the time interval in which diabetes thereafter developed in the animal, I have attempted to produce a composite concept of the sequence of pancreatic events which lead to the development of IDDM in the BB/E rat.

Table XI relates the time interval between the appearance of insulitis in pancreatic biopsies of individual animals, and the subsequent development of diabetes in those animals. Twenty two of the 40 study animals (55%) developed IDDM with the mean age at onset of diabetes occurring at 102 ± 29 days (mean \pm SD). Intervals between the time of the last pancreatic biopsy performed in the prediabetic period, and the subsequent time of diabetes onset, ranged from 7 to 130 days.

With one exception, insulitis was never observed in biopsies which preceded the onset of diabetes by more than three weeks. Between 2 to 3 weeks before the onset of diabetes, although insulitis was occasionally found in some animals, the major pancreatic development was the appearance of infiltrates at periductal, perivascular and peri-islet sites. Biopsies taken one to two weeks before disease onset, consistently showed extensive and intensive infiltration of the islets. These results suggest that in the majority of cases, the onset of diabetes in the BB/E rat is a rapid process occurring within two weeks of the initial insulitis.

Monoclonal antibodies against lymphocyte and macrophage subsets, were used to characterize the composition of these pancreatic infiltrates and MoAbs against rat MHC molecules were used to further document the expression of MHC antigens in the prediabetic and diabetic BB/E rat pancreas.

Fig 5 : Double exposure of OX-6 (green) and insulin (red) staining on DP BB/E rat pancreas (aged 30 days). Shows extent and distribution of MHC class II positive cells in the normal non-diabetic pancreas. X125

Fig 6 : OX6 staining revealing MHC class II expression on vascular endothelium of DP BB/E rat pancreas (aged 45 days). X640

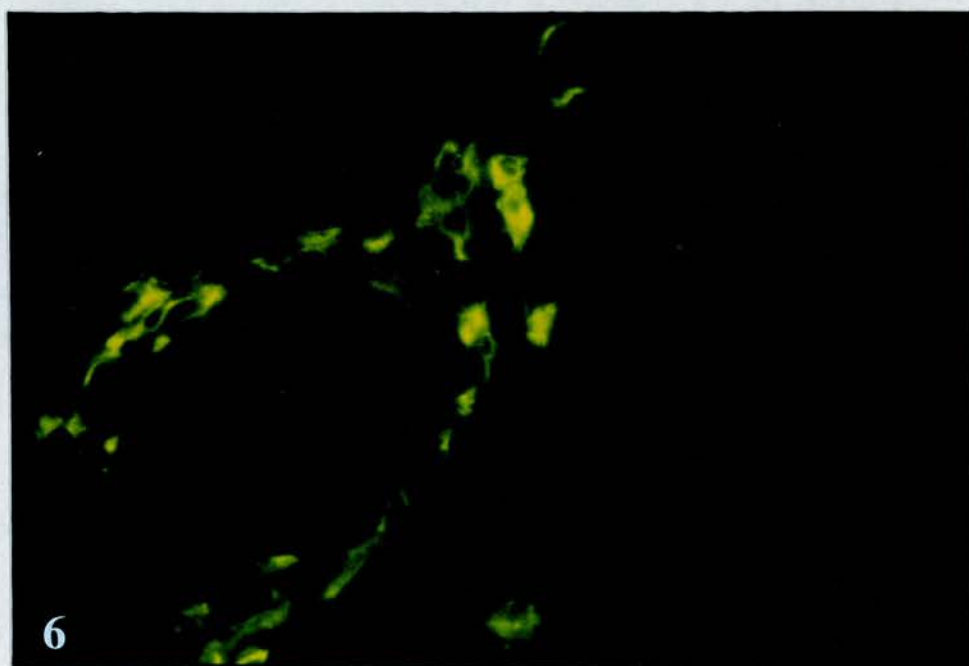
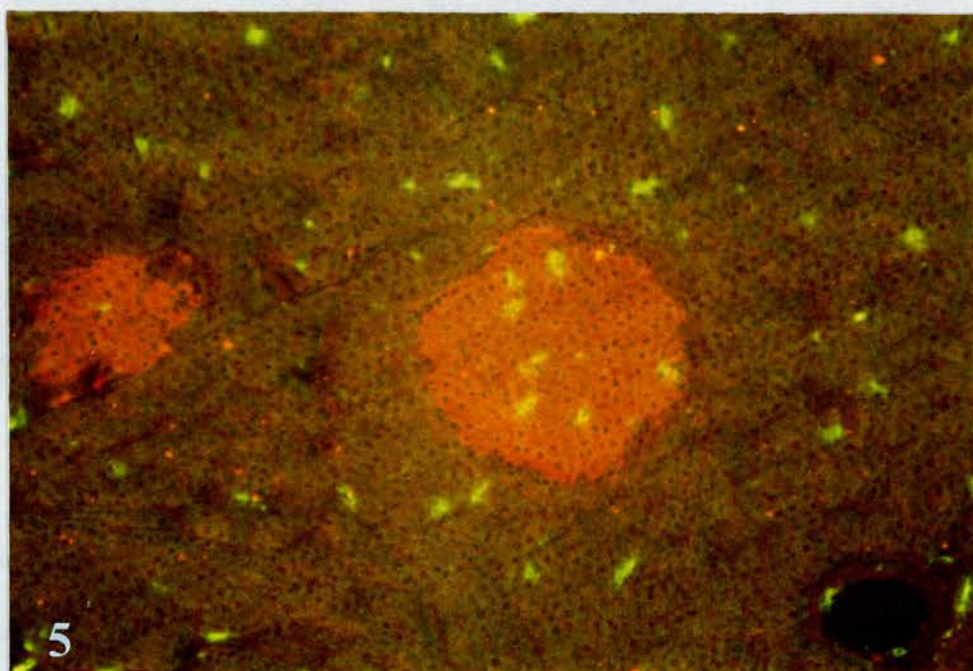


TABLE X: PANCREATA WITH INCREASED MHC CLASS II EXPRESSION
ON NON LYMPHOID CELLS AND THOSE WITH INSULITIS
(STUDY I)

DP BB/E RATS (age in days)	NORMAL	INCREASED NON LYMPHOID Ia expression (% positive per group)	INSULITIS
30 (N=12)	100	0	0
45 (N=12)	58	42	0
60 (N=12)	42	50	8
75 (N=9)	33	44	22
90 (N=11)	36	9	54
105 (N=11)	18	18	64
Total DP BB/E (N=67)	49	27	24
Total DR BB/E (N=22) (30-105 days)	64	32	4

Fig 7 : Sequential sections from a 105 day old
DP BB/E rat with insulinitis. X 400

- (a) W3/25 (anti-T_H and some macrophages).
- (b) double exposure for W3/25 (red) and
OX6* (green).
- (c) OX-8 (anti-Tcyt/supp and NK cells).
- (d) OX-12 (anti-Ig kappa chain).

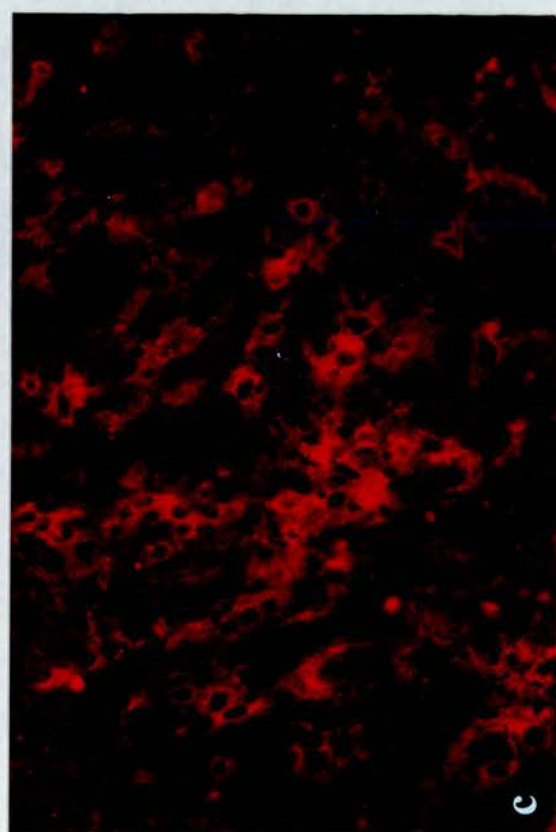
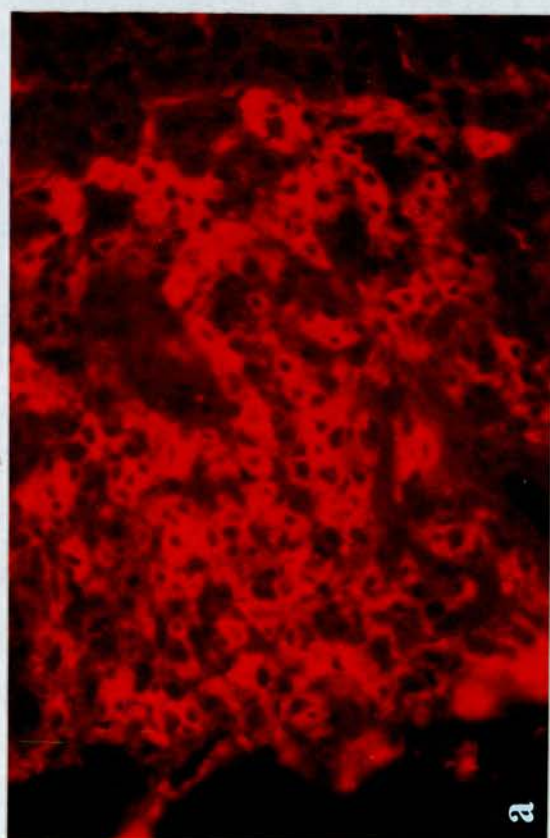
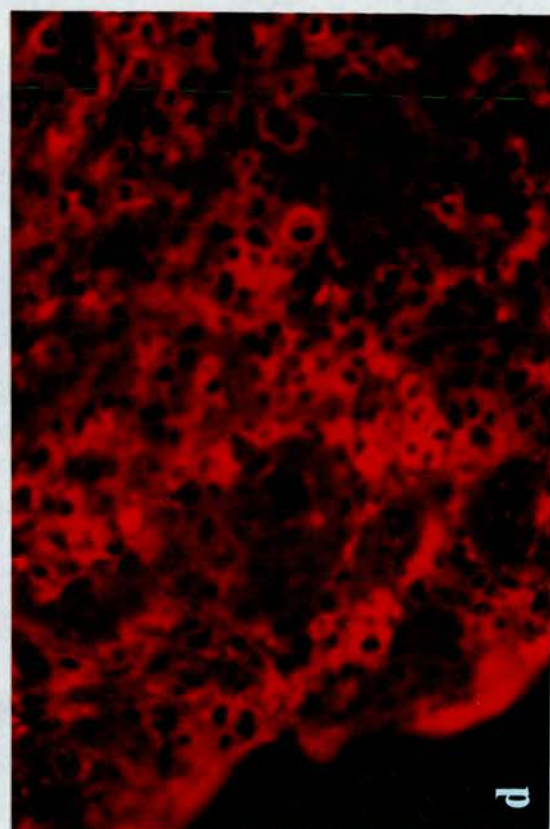
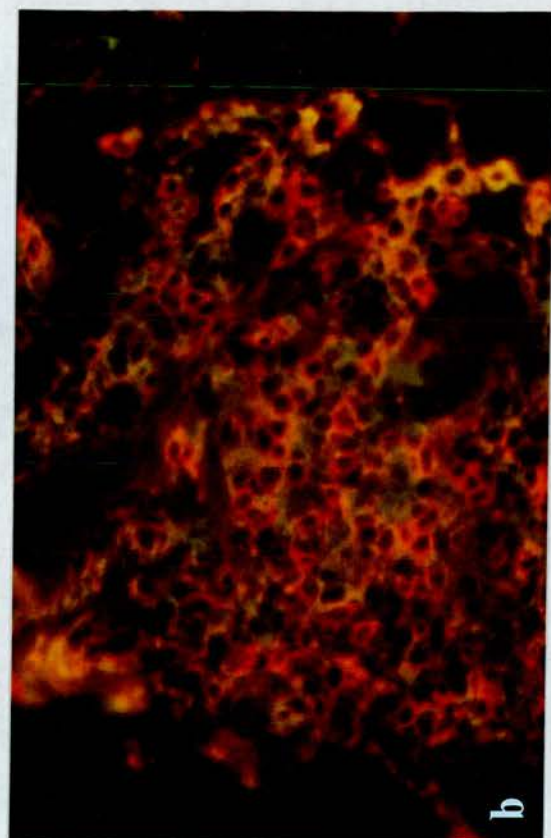


Fig 8 : DP BB/E rat pancreas (75 days old). X640

- (A) OX-6 staining reveals insulinitis and class II MHC expression on "endocrine-like" cells.
- (B) anti-insulin staining (M1183) on serial section.
- (C) double exposure for insulin and OX-6 indicates expression of MHC class II proteins on insulin-containing cells.

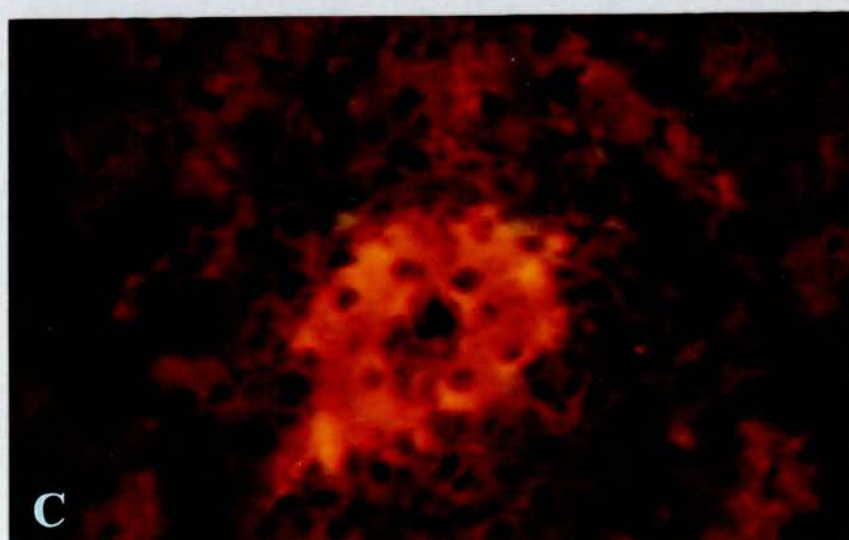
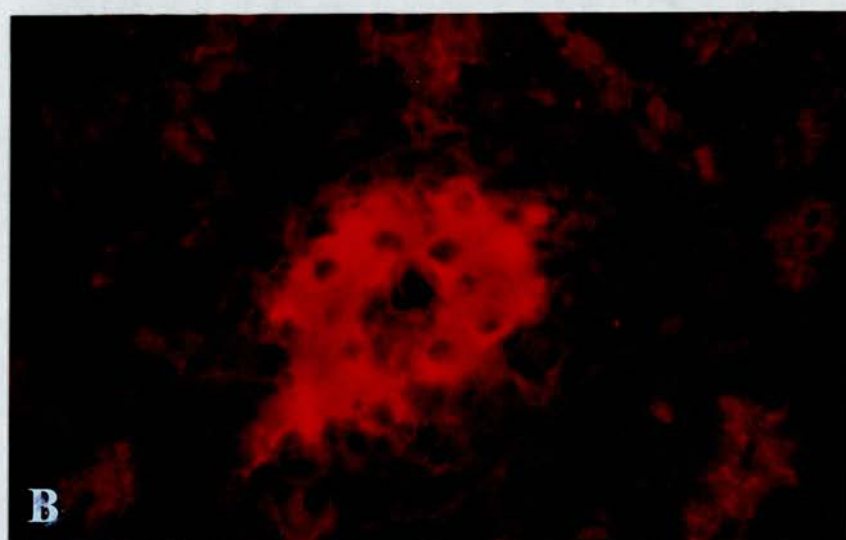
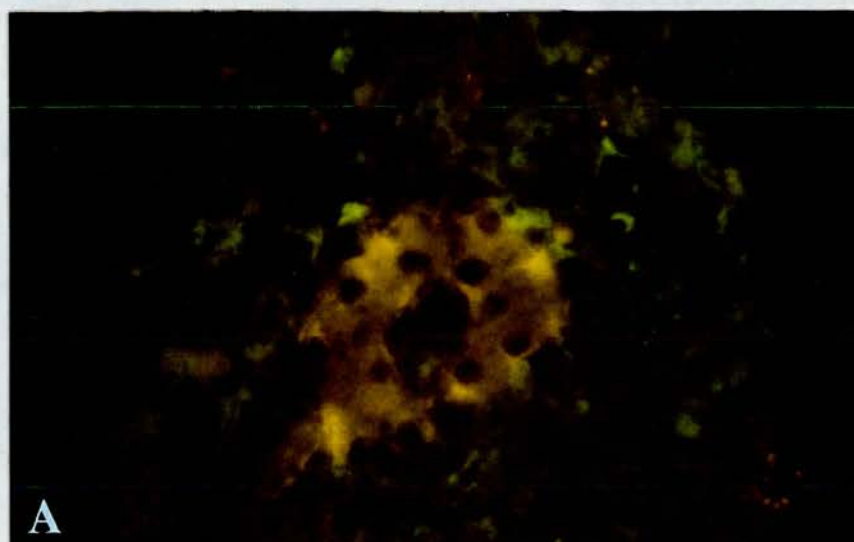
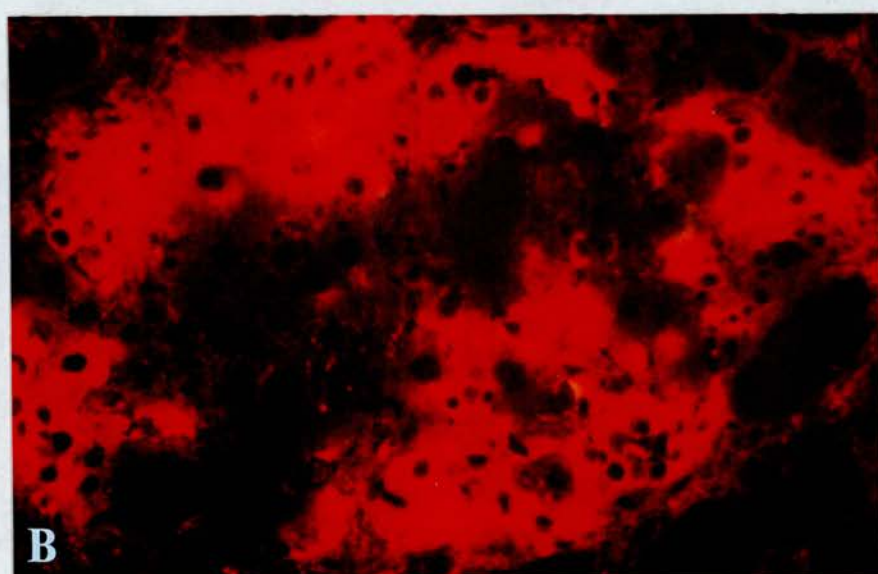
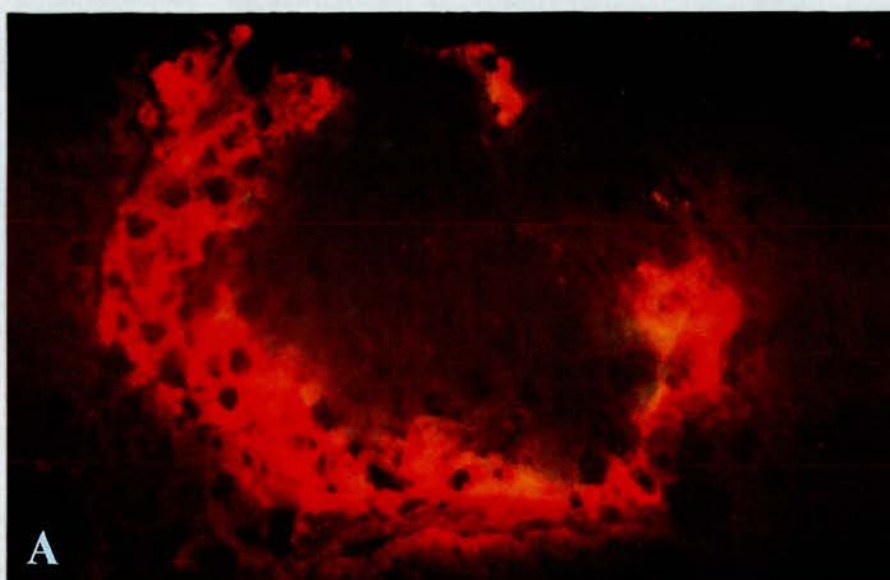


Fig 9 : Double exposure for glucagon-containing cells (red) and insulin-containing cells (green).

- (A) Normal islet with glucagon cells at islet mantle and insulin cells at islet core. (DP BB/E rat aged 60 days). X312
- (B) Islet from DP BB/E rat aged 105 days. Showing selective destruction of insulin-containing cells. X500



The first change observed in the biopsies of prediabetic DP BB/E rats, was seen in some biopsies taken from 40 and 50 day old rats and consisted of an increase in the expression of MHC class I molecules on the vascular endothelium and on islet cells within some pancreatic lobules (Fig. 10). The increase in intra-islet class I expression appeared to initially occur on the beta cells at the islet core and gradually spread to the adjacent cells to include the glucagon cells at the islet mantle. This increased expression of class I molecules occurred in the absence of insulitis which has never been observed in any animal before 60 days of age (Table X, Table XI). Although there is variation in the time scale of pancreatic changes within individual animals, this altered class I expression could be detected onwards of 30 days prior to the onset of IDDM.

The next phase of altered pancreatic morphology was represented by the beginnings of pancreatic infiltration coincident with the expression of MHC class II molecules on endothelial cells of some ducts and vessels.

As already mentioned and indicated in Table XI, this phase of pancreatic infiltration occurs 2-3 weeks before the onset of diabetes and is characterized by the presence of infiltrates at periductal and perivascular sites (Fig. 11a). Analysis of the infiltrates revealed that the majority of infiltrating cells at this stage were identifiable with the MoAb ED1 which recognizes a population of macrophages (Fig. 11b).

In the pancreas of normal Wistar rats, DR BB/E rats and young (30-40 day old) DP BB/E rats, ED1-positive cells were infrequently observed (Fig. 12a). This is in contrast to another population of macrophages which are recognized by the MoAb ED2 and which were much more numerous than ED1⁺ cells in the control pancreata.

- Fig 10 : (a) Normal class I MHC antigen expression (non-diabetic DP BB/E rat aged 50 days). Islet (i) is negative whilst endothelial cells of an adjacent blood vessel are positive (arrows). X 312
- (b) Higher magnification of islet in (a). X 625
- (c) DP BB/E rat pancreas (aged 50 days) showing increased expression of class I MHC molecules on the vascular endothelium (arrows) and within the adjacent non-infiltrated islet (i). (Biopsy taken 28 days prior to onset of IDDM). X 312
- (d) Higher magnification of islet in (c). X 625

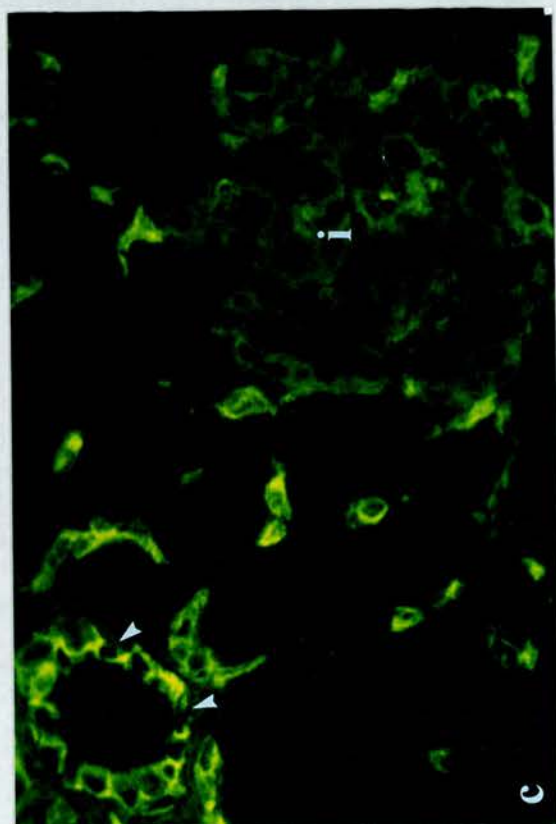
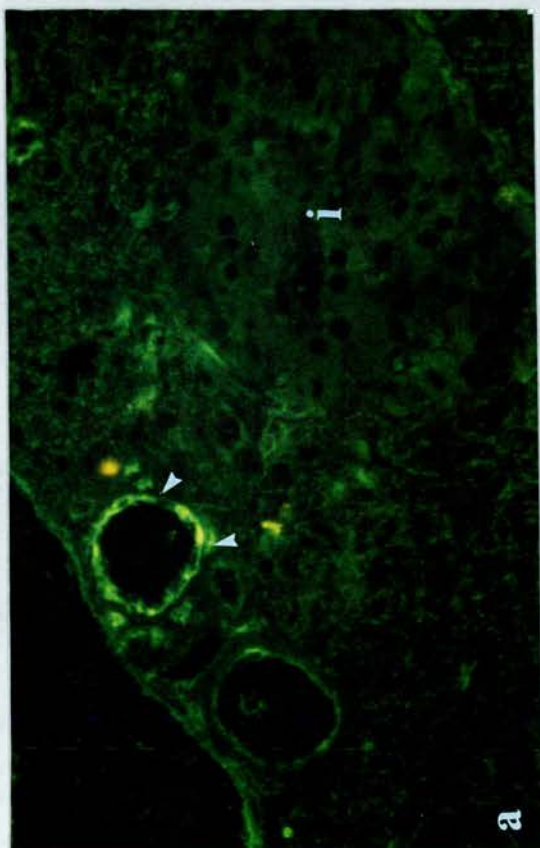
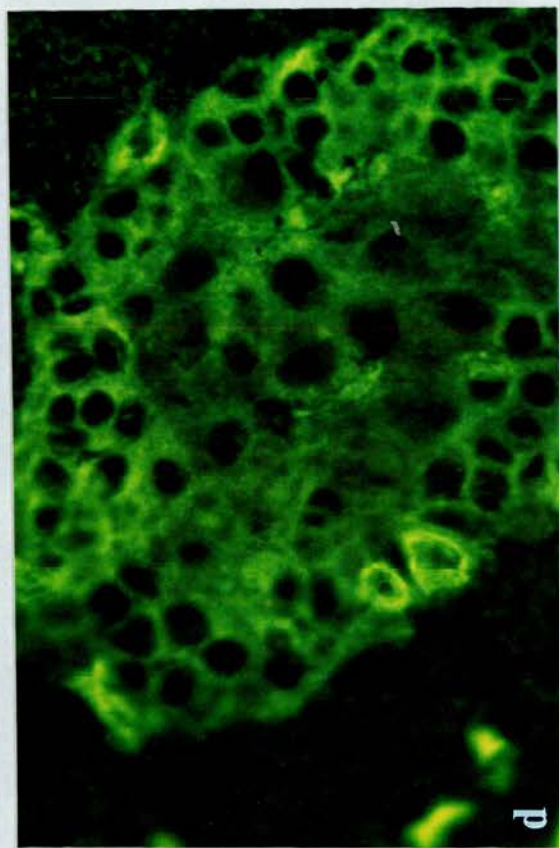
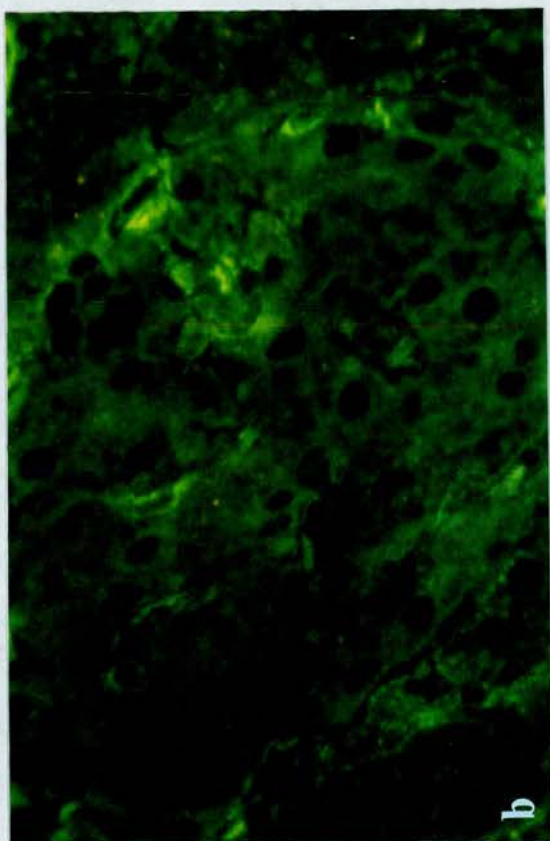


Fig 11 : DP BB/E rat aged 100 days. Biopsy taken
18 days before onset of diabetes. X 312

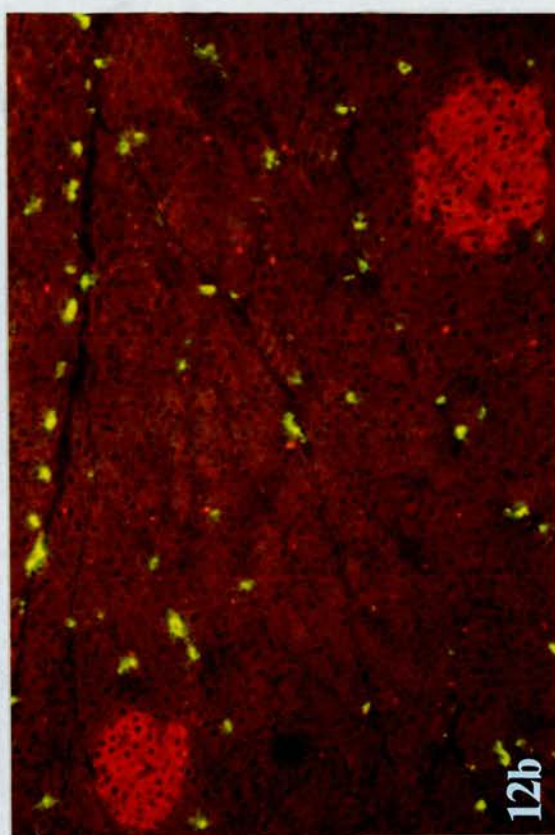
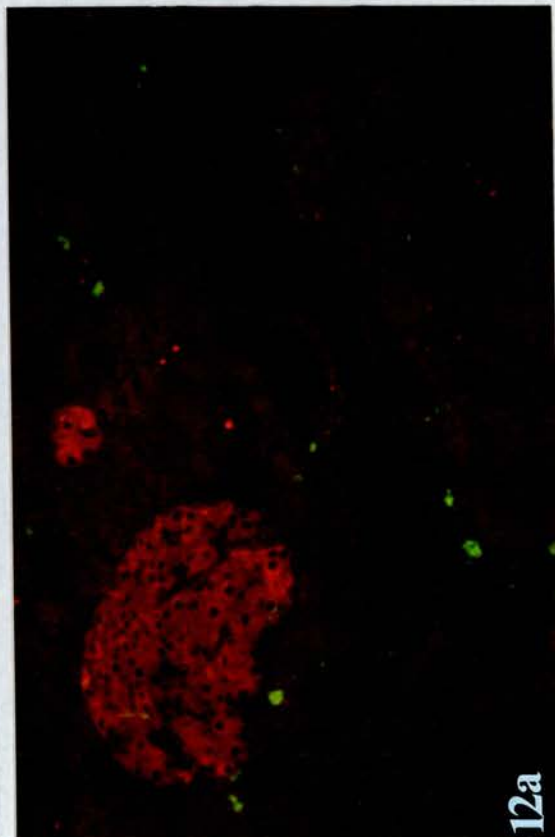
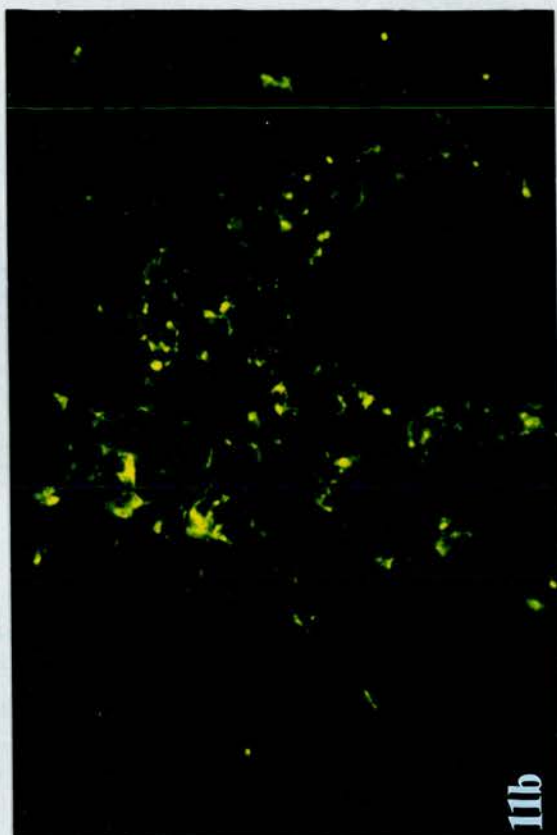
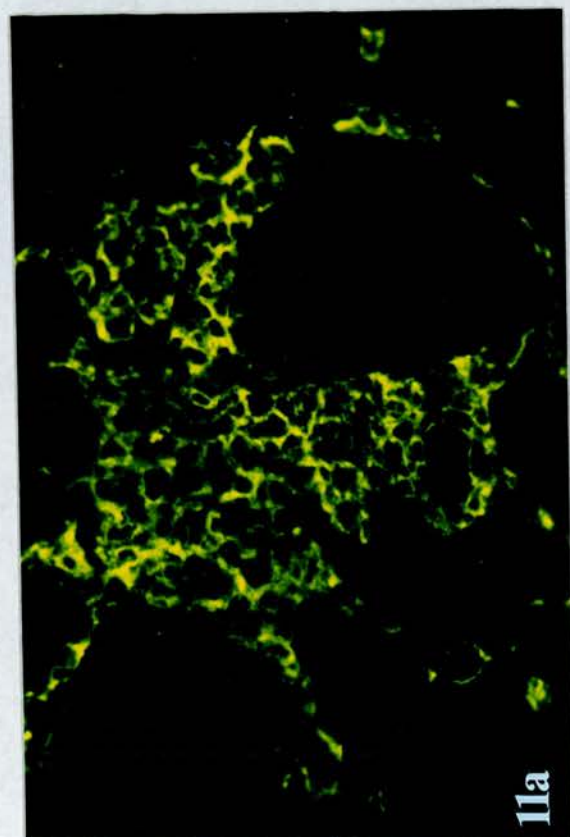
(a) OX-6 staining showing perivascular
infiltrates of MHC class II positive
cells and expression of class II MHC
antigens on some vascular endothelial
cells.

(b) ED1 staining on serial section.

Fig 12 : Normal Wistar rat pancreas (aged 100 days).
X 125

(a) double exposure for ED1 (green) and
insulin (red).

(b) double exposure for ED2 (green) and
insulin (red). ED2⁺ cells are
prominent within septa of pancreatic
lobules.



Studies revealed that ED2 MoAb identified a resident population of tissue macrophages present in all biopsies from all groups of rats. These cells were prominent, lining the septa between pancreatic lobules and were widely dispersed throughout the connective and acinar tissues but were not present within pancreatic islets (Fig. 12b). Fluctuations in the degree of dispersion and number of ED2⁺ cells, found between sequential biopsies in individual rats, were minimal and may have been a consequence of the biopsy procedure. Biopsies from DP BB/E animals at all ages, showed a similar tissue distribution irrespective of the presence or absence of insulinitis.

Figure 13a graphically illustrates the early stages of pancreatic infiltration where many MHC class II bearing cells are seen accumulated around the pancreatic ducts and blood vessels. The cells then seemingly home to the nearby islets where cuffings of peri-islet infiltrates are formed (Fig. 13c). Staining of serial sections revealed that most of the class II-positive infiltrating cells were ED1⁺ macrophages (Fig. 13b, Fig. 13d). In addition, class II expression on endothelial cells, of ducts and vessels as well as capillary cells within both the endocrine and exocrine tissue, was observed. However, at this stage of pre- or early insulinitis, expression of class II MHC molecules was not observed on islet beta cells (Fig. 13a, Fig. 13c). Initially, this infiltration could be quite focal whereby most islets within a few pancreatic lobules were affected but islets in other areas were unaffected (Fig. 13a, Fig. 13e). This initial accumulation of ED1⁺ cells, at perivascular locations, was not a non specific consequence of the biopsy procedure since it was never observed following similar serial biopsies performed on control normal Wistar or DR BB/E rats. Subsequent to their recruitment and accumulation at peri-islet sites, the ED1⁺ cells

then proceeded to infiltrate the islets within the affected lobules (Fig. 13f), and this appeared to occur before there was any apparent involvement of other immune effector cells.

Following this initial infiltration, the development of insulitis and its severity progressed as other immune effector cells were recruited. As indicated by Table XI, the height of the autoimmune reaction against the islet beta cells appeared to occur approximately 10 days before the onset of IDDM. Therefore, in an attempt to quantitate the cell populations contributing to the insulitis, biopsies taken 10-15 days prior to the onset of diabetes were screened.

Table XII shows the number of each subset of cells infiltrating individual islets with different degrees of insulitis severity, examples of which are illustrated in Figures 3 and 14. Marked numbers of T lymphocytes (OX19⁺) were observed only in islets showing more advanced stages of insulitis, where a significant loss of insulin staining beta cells was also observed (Fig. 14c). In all cases, within infiltrated islets irrespective of the degree of insulitis, cells recognized by the ED1 MoAb were the most numerous, being at least twice as frequent as cells recognized by the OX-19 MoAb. The greater number of W3/25⁺ cells when compared to the number of OX-19⁺ cells, is not unexpected since many of the W3/25⁺ cells also express the ED1 marker. However, although double labelling for the ED1 and W3/25 antigens has not been done, from staining of serial sections 75-80% of the ED1⁺ cells are also recognized by the W3/25 marker. It appeared that most of the OX-19⁺ T cells were however of the T_H phenotype (Fig. 15c, Fig. 15d), and that some of the OX-8⁺ cells did not express the OX-19 antigen suggesting that they were NK cells (Fig. 16c, Fig. 16d).

TABLE XI: TIME COURSE ON THE APPEARANCE OF INSULITIS PRIOR TO
THE SUBSEQUENT DEVELOPMENT OF IDDM (STUDY II)

Rat (N=22)	Sex	Last previous biopsy	Onset of diabetes	Time interval (days)	Insulitis
		Age of rat (days)			
1	M	60	190	130	Absent
2	M	60	170	110	Absent
3	M	50	108	58	Absent
4	F	60	108	48	Absent
5	M	50	98	48	Absent
6	F	50	94	44	Absent
7	F	50	94	44	Absent
8	M	90	123	33	Absent
9	F	70	101	31	+
10	F	50	80	30	Absent
11	F	60	83	23	+/-
12	M	80	101	21	++
13	M	90	108	18	+
14	F	100	118	18	+/-
15	M	80	96	16	+/-
16	F	70	83	13	+/-
17	F	70	83	13	+
18	M	90	101	11	++
19	F	60	70	10	++
20	F	80	90	10	++
21	M	60	68	8	+
22	F	70	77	7	++

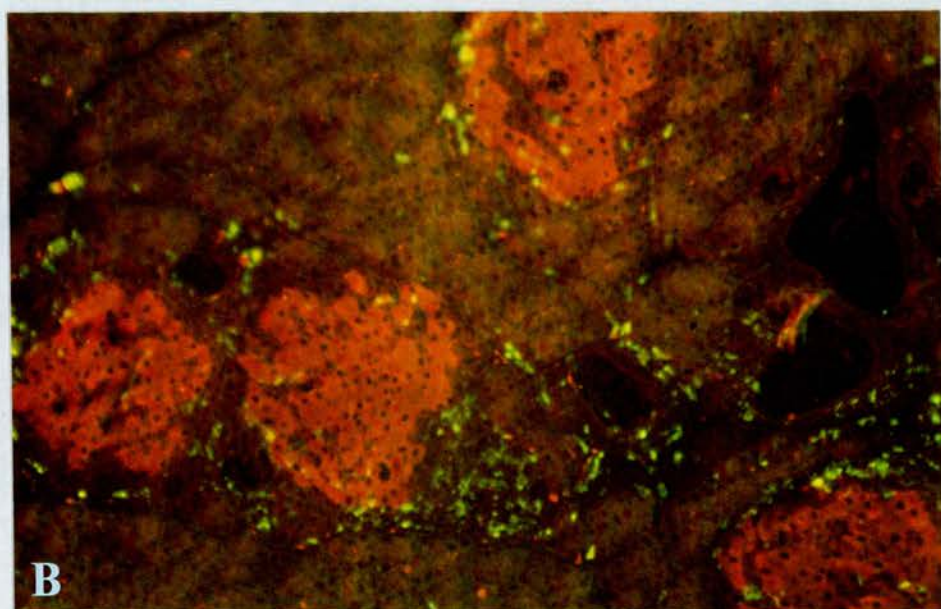
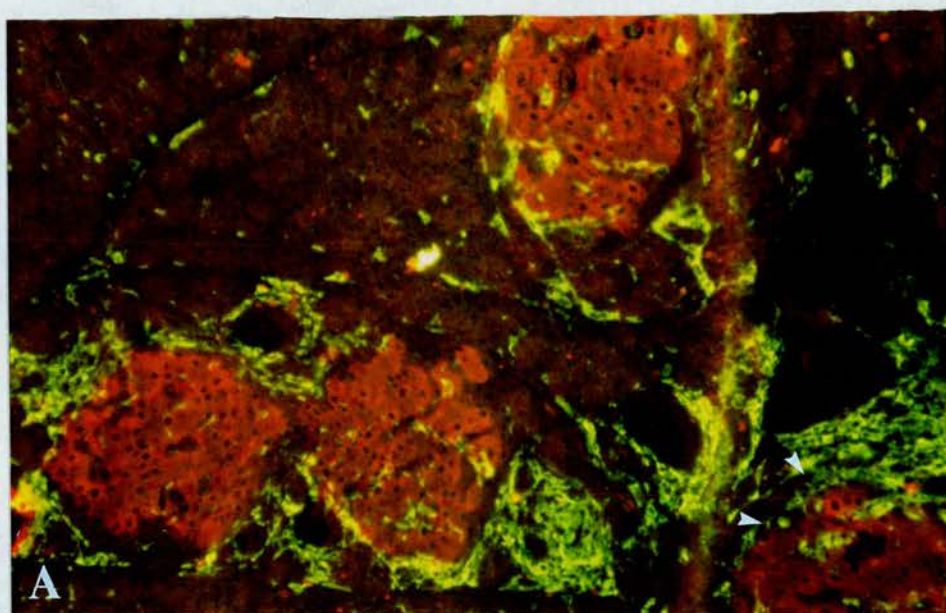
+/- : Pancreatic infiltration at perivascular and peri-islet sites.

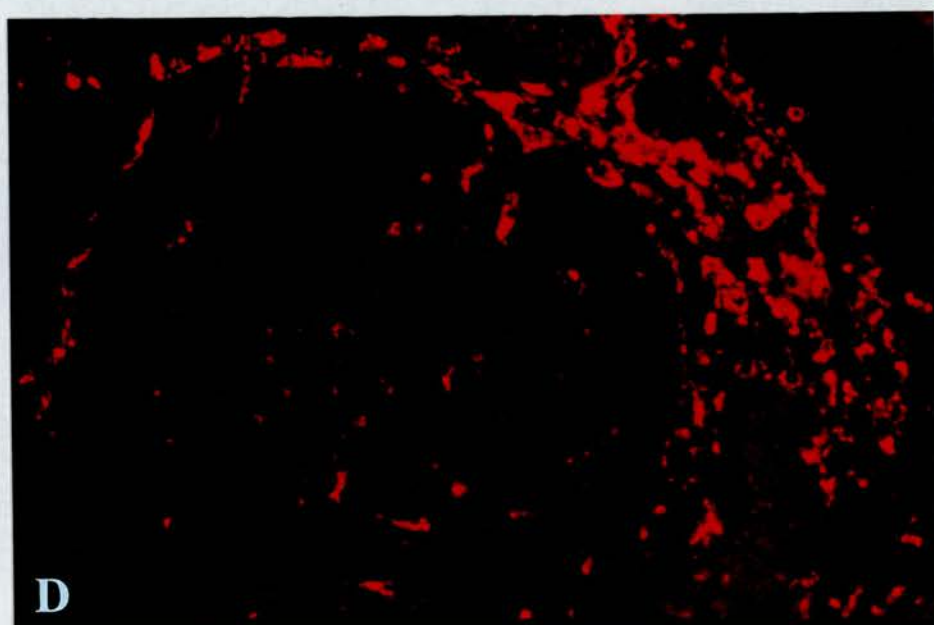
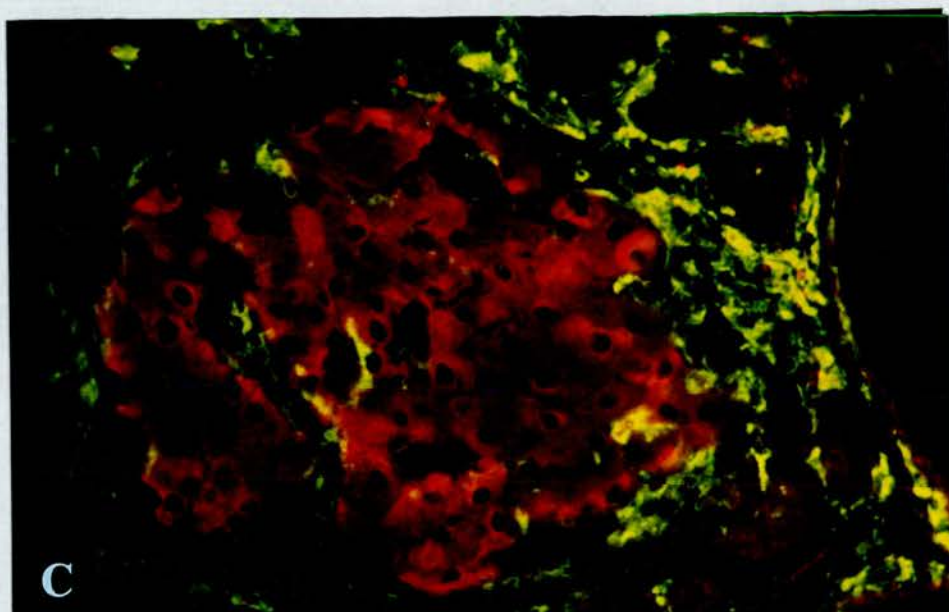
+ : Insulitis affecting $\frac{1}{3}$ - $\frac{1}{2}$ of 1-5 islets.

++ : Insulitis extensive over 5 or more islets.

Fig 13 : A - D; serial sections from biopsy of DP BB/E rat (aged 70 days). Biopsy was taken 20 days before onset of diabetes.

- (A) double exposure for cells expressing MHC class II molecules (green) and insulin (red). X 125
- (B) serial section to (A) stained for ED1 (green) and insulin (red) shows many of the OX6⁺ cells to be ED1⁺ macrophages. X 125
- (C) OX-6 (green) and insulin (red) staining at higher magnification (X 312) of islet marked with arrows in (A).
- (D) serial section of (C) stained for ED1.
- (E) OX-6 (green) and insulin (red) staining of another area from the same biopsy as A-D above, reveals no infiltration, and emphasizes the "patchy" heterogeneous histopathology of the disease. X 125
- (F) double exposure of ED1⁺ cells (green) infiltrating insulin-containing islet (red). Biopsy taken from same animal 10 days later from sections shown in A-E. X 312





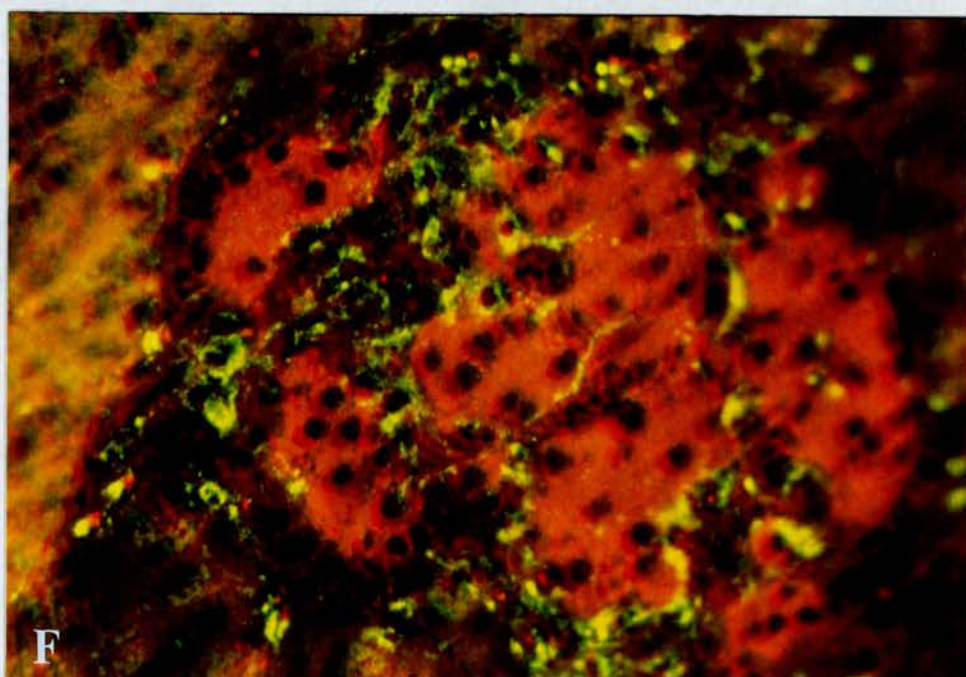
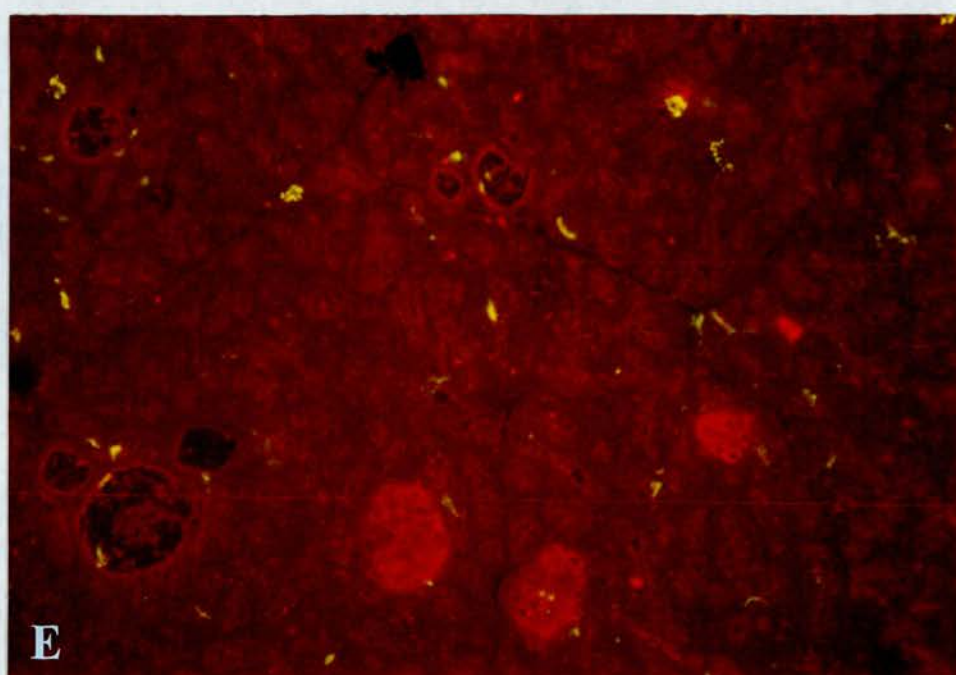


TABLE XII: QUANTITATIVE ANALYSIS OF INSULITIS IN DP BB/E RATS :
BIOPSIES 10-15 DAYS PRIOR TO ONSET OF DIABETES

Degree of insulitis	NUMBER OF INFILTRATING CELLS (MEAN \pm SEM) / ISLET					
	OX-6	ED1	W3/25	OX-19	OX-8	OX-33
+ (N=12)	65 \pm 2.1	43 \pm 2.3	44 \pm 3.4	13 \pm 0.8	10 \pm 0.7	1 \pm 0.4
++ (N=16)	122 \pm 3.2	72 \pm 2.8	71 \pm 4.2	38 \pm 1.7	28 \pm 1.8	5 \pm 1.0
+++ (N=18)	200 \pm 8.9	99 \pm 7.4	113 \pm 12.3	52 \pm 5.0	42 \pm 3.9	31 \pm 3.1

+ : 50-100 OX-6⁺ cells; Insulitis mainly at islet periphery;
 - Majority of beta cells intact.

++ : 100-150 OX-6⁺ cells; Insulitis affecting $\frac{1}{3}$ - $\frac{1}{2}$ of islet;
 Loss of $\frac{1}{2}$ - $\frac{2}{3}$ beta cells.

+++ : >150 OX-6⁺ cells; Insulitis affecting most of islet;
 Virtual absence of beta cells.

Fig 14 : Serial sections from biopsy of DP BB/E rat (aged 90 days). Biopsy was taken 11 days before onset of diabetes. X 125

- (A) double staining for MHC class II antigen positive cells (green) and insulin (red). Three islets with increasing severity of insulinitis and accompanying reduction of residual insulin are shown.
- (B) ED1 staining on serial section of (A).
- (C) OX-19 (pan T cell) staining on serial section of (A).

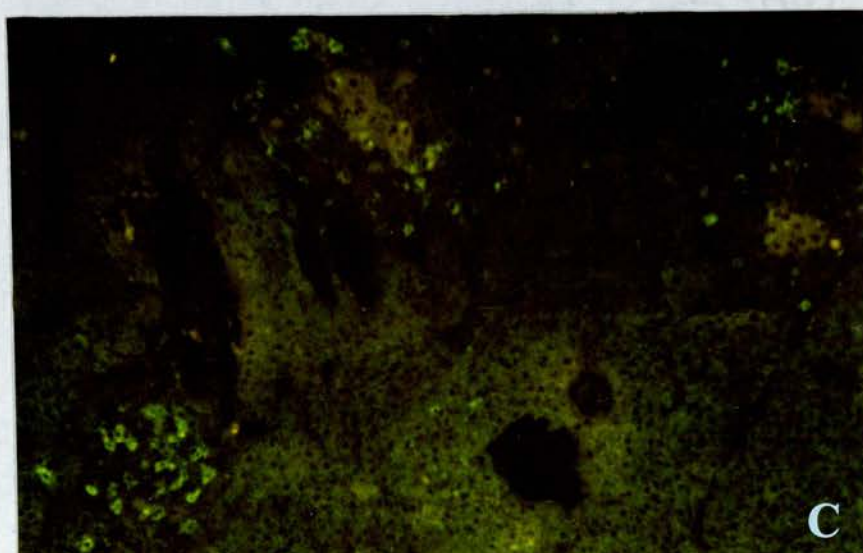
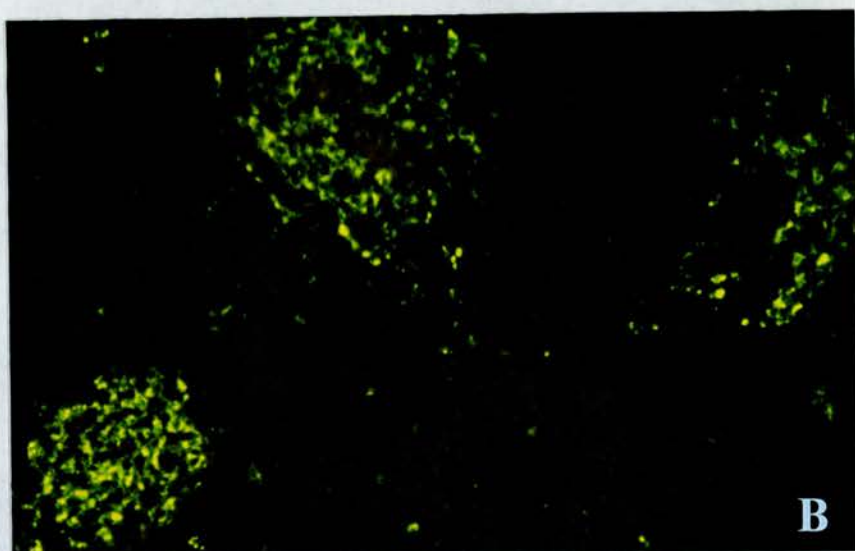
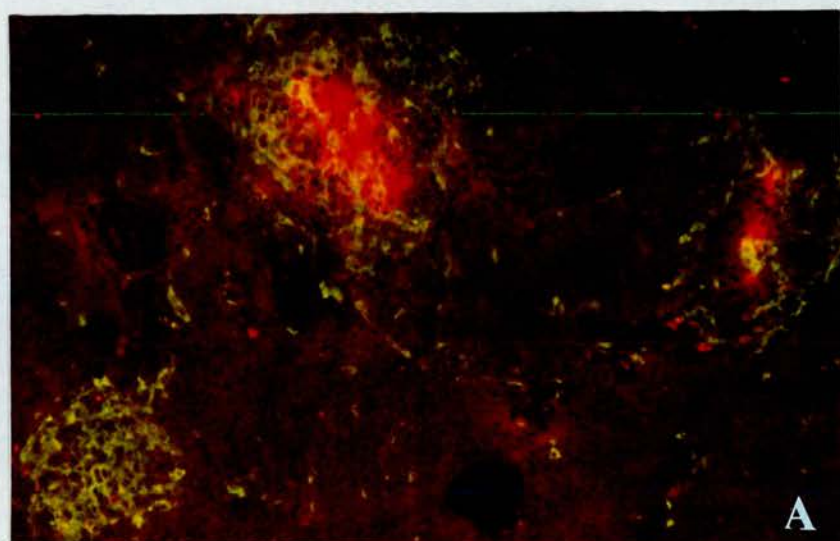


Fig 15 : Serial section staining of insulinitis (+++) in islet from DP BB/E rat (aged 90 days). Biopsy was taken 11 days before onset of diabetes. X 312

- (a) MHC class II positive cells
- (b) double exposure for ED1 (red) and insulin (green) on serial section of (a).
- (c) OX-19 (pan T cells) staining on serial section of (a).
- (d) W3/25/OX-35 (T_H cell and macrophage) staining on serial section of (a).

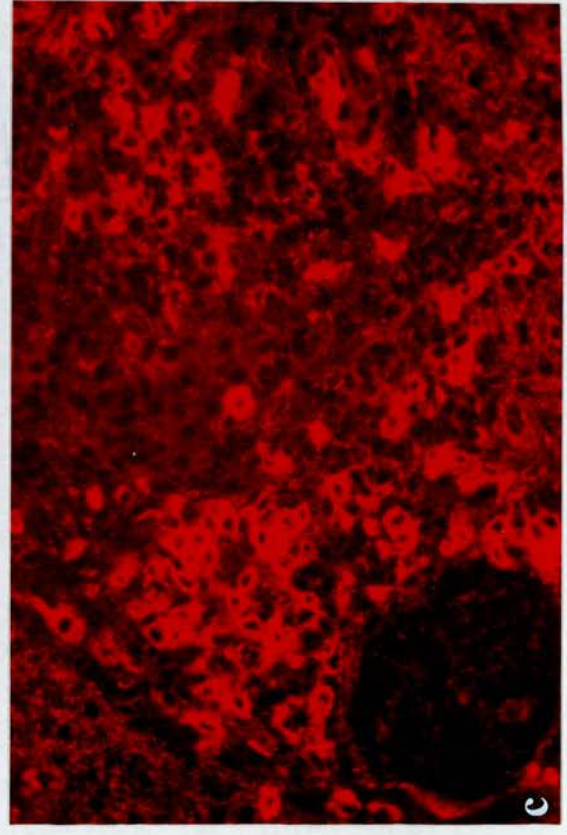
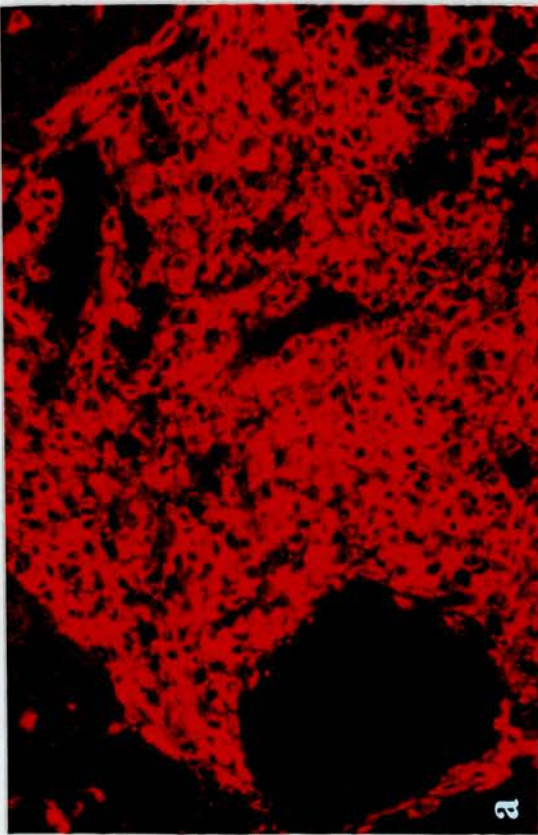
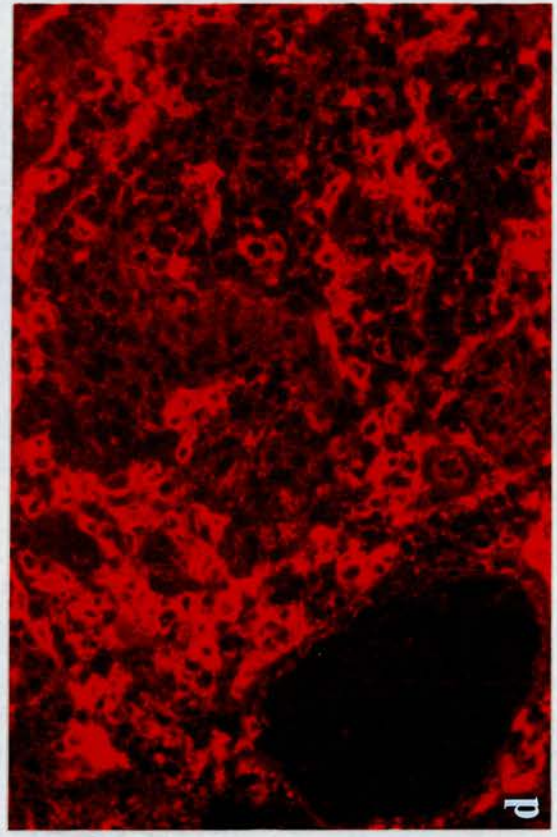
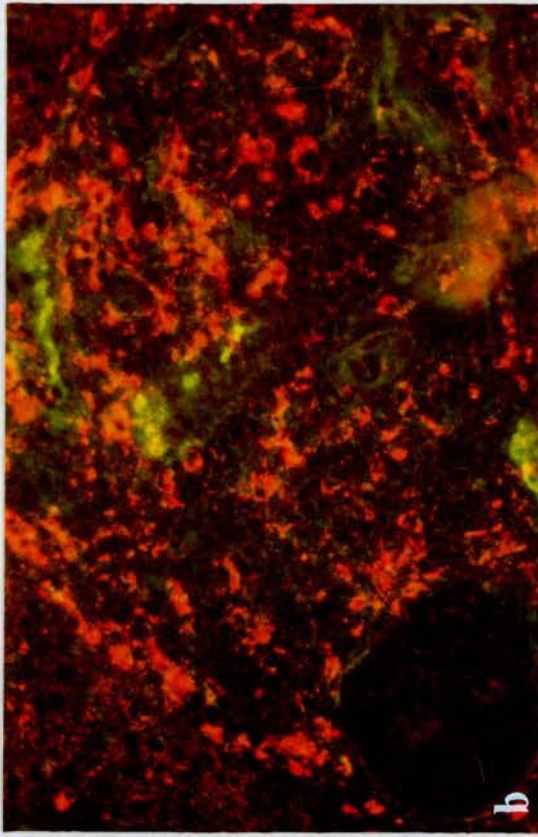
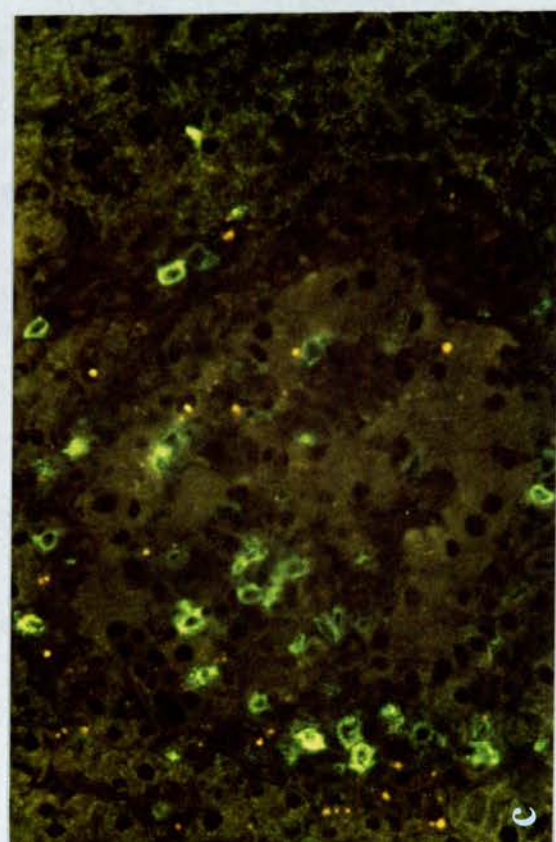
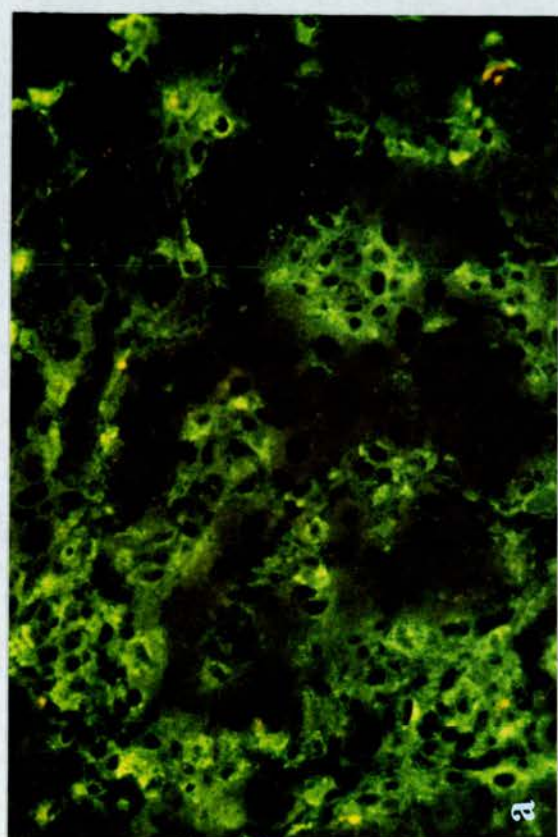
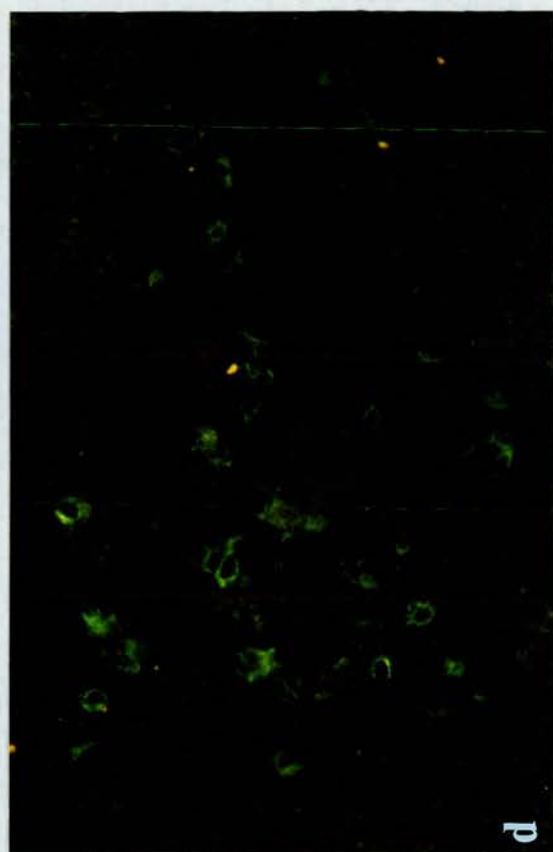
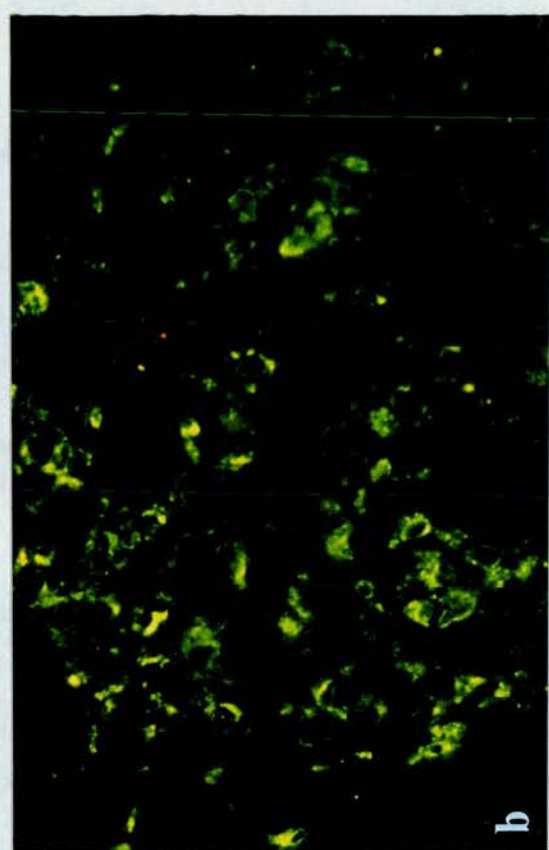


Fig 16 : Serial section staining of insulinitis (++)
in islet from DP BB/E rat (aged 90 days).
Biopsy was taken 11 days before disease
onset. X 312

- (a) MHC class II positive cells
- (b) ED1 positive cells
- (c) pan T cell staining
- (d) OX-8 (T cyt/supp cells and NK cells)
staining



Significant numbers of B lymphocytes (OX-33⁺) only appeared in islets showing a marked loss of insulin producing cells, thereby indicating they appear late in the disease process.

At this stage in the disease process, hyperexpression of MHC class I antigens, within infiltrated islets, is markedly enhanced. This enhanced expression of class I molecules is no longer confined to the islet endocrine cells but extends beyond the islet periphery into the surrounding exocrine tissue, until once again the lobular architecture of the pancreas appears to create a physical barrier to this phenomenon (Fig. 17). At this point in the study, within the infiltrated islets, the expression of class II molecules on insulin containing cells was rarely seen with class II expression being restricted to the infiltrating immunocytes (Fig. 18). Progressive spreading of this insulitis process, from lobule to lobule with resultant beta cell loss, will eventually result in the onset of overt diabetes.

Biopsies taken at onset of diabetes, contained some islets with residual insulin containing cells which also hyperexpressed class I molecules in the presence of insulitis (Fig. 19a-c). Other islets however no longer retained an infiltrate and appeared as end stage islets where the glucagon cells, in the absence of the beta cell core, collapse inwards to form a clump (Fig. 19d). In biopsies taken from animals developing ketoacidosis, virtually all of the insulin containing islets had disappeared and consequently insulitis was rarely observed. Most islets were end stage in character with occasional ED1⁺ cells remaining around the collapsed islets (Fig. 20).

Hyperexpression of MHC class I molecules was no longer observed in end stage islets and was only occasionally observed in areas where small foci of class II bearing infiltrates remained.

Figure 21 describes, in a flow diagram presentation, the sequence of pancreatic events that may lead to the development of IDDM in the BB/E rat. It is recognized that some of the DP BB/E animals remaining non diabetic or even some of the DR BB/E animals, may show some of these individual features but not of sufficient severity to result in overt disease.

Fig 17 : DP BB/E rat pancreas (aged 70 days). Biopsy taken 7 days before onset of diabetes.

- (A) OX-18 staining, showing hyperexpression of MHC class I molecules accompanying insulitis in some pancreatic lobules, whilst adjacent pancreatic lobules are normal. X 50
- (B) Higher magnification (X 312) of one of the islets (i) in (A), shows hyperexpression of MHC class I molecules extends into the surrounding exocrine tissue. (arrows indicate the islet boundary).

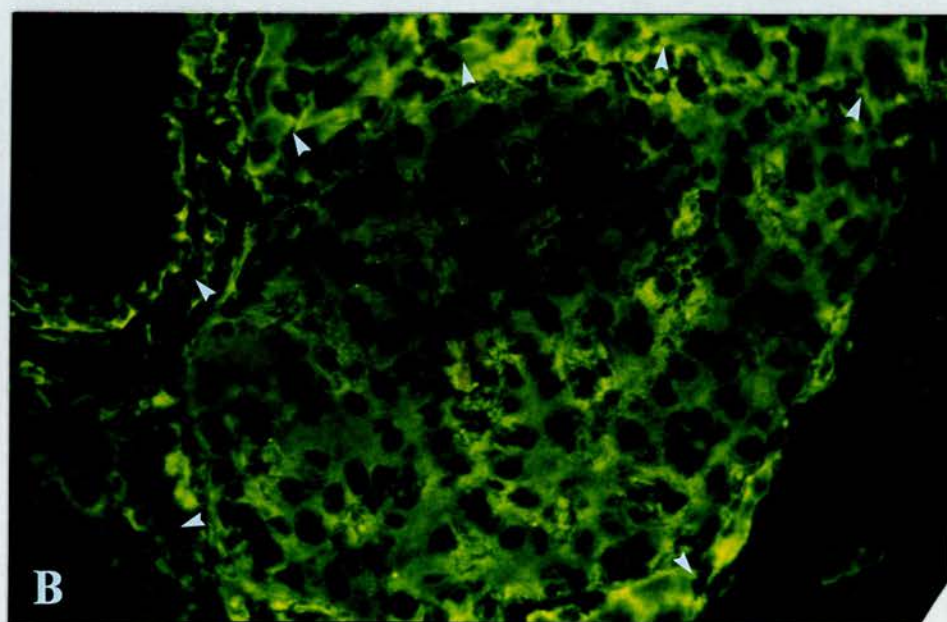
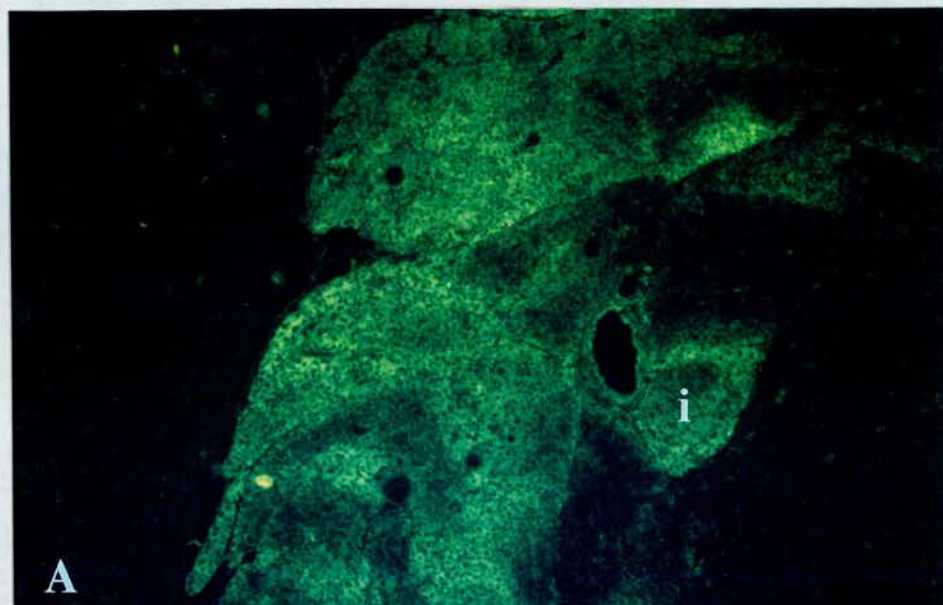


Fig 18 : DP BB/E rat pancreas (aged 90 days). Biopsy taken 11 days before onset of diabetes.

(A) & (B)*double staining for MHC class II positive cells (green) and insulin (red) reveals expression of Ia antigens only on the infiltrating leucocytes. X 625 & X 312 respectively.

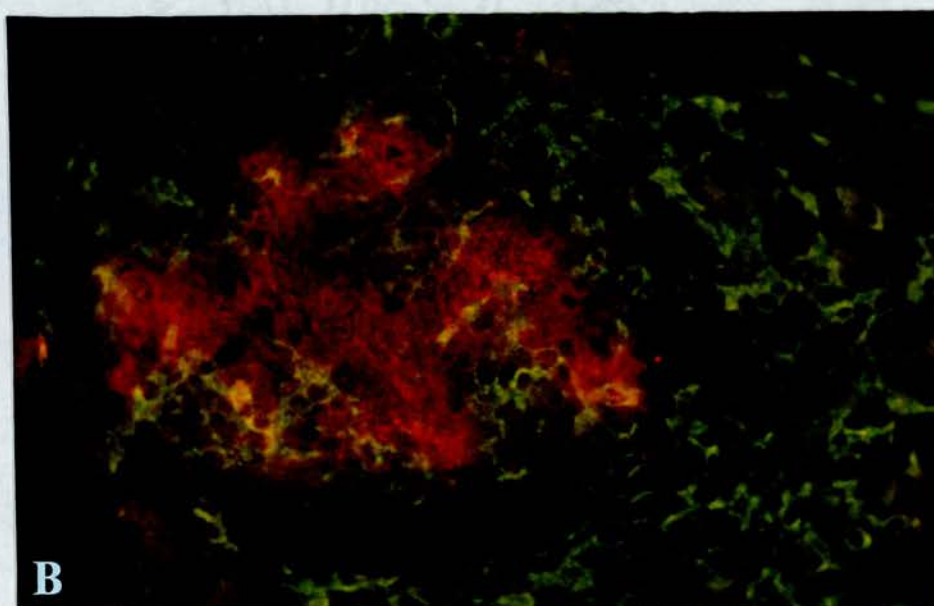
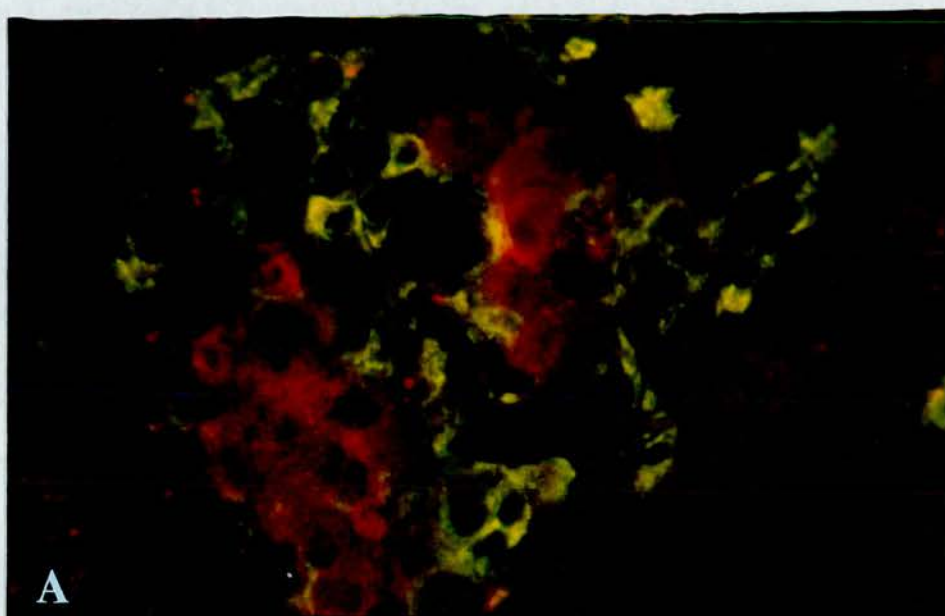


Fig 19 : DP BB/E rat pancreas at onset of diabetes
(aged 101 days).

- (a) OX-18 staining showing hyperexpression of MHC class I molecules within an islet retaining both infiltrating leucocytes and residual insulin-containing cells. X 312
- (b) ED1 staining on serial section of (a).
- (c) Insulin staining (M1183) on serial section of (a).
- (d) glucagon staining of another islet within same biopsy shows "end stage" characteristic clumping of intact glucagon cells. X 500

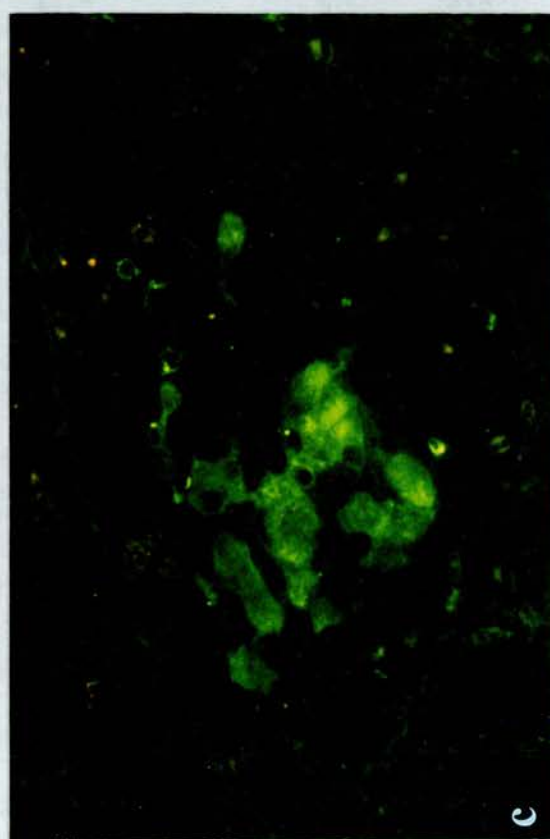
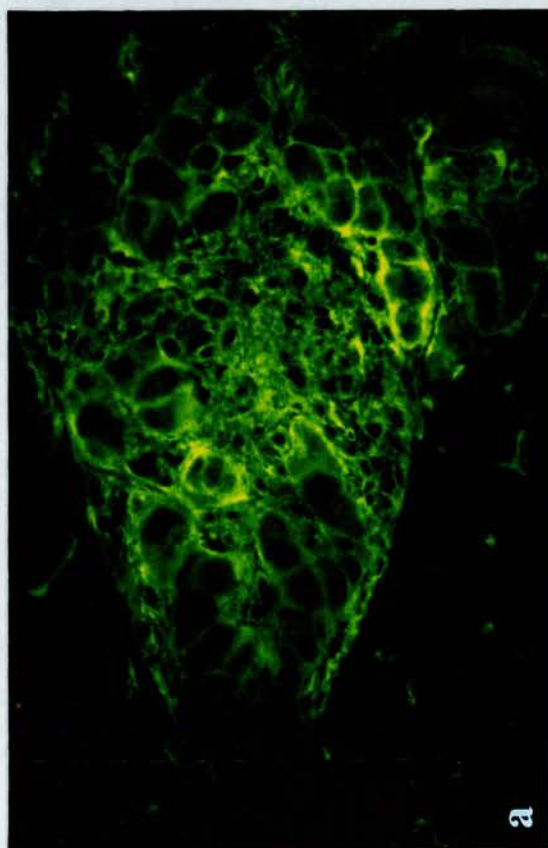
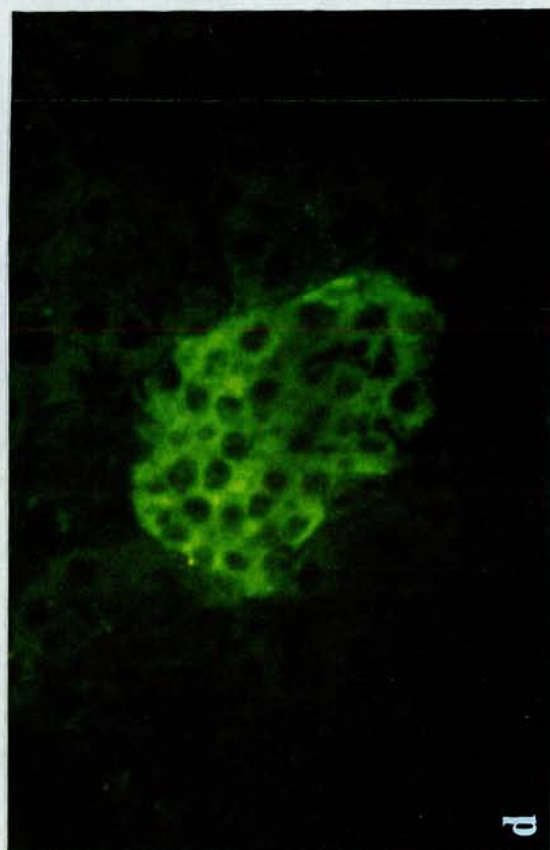
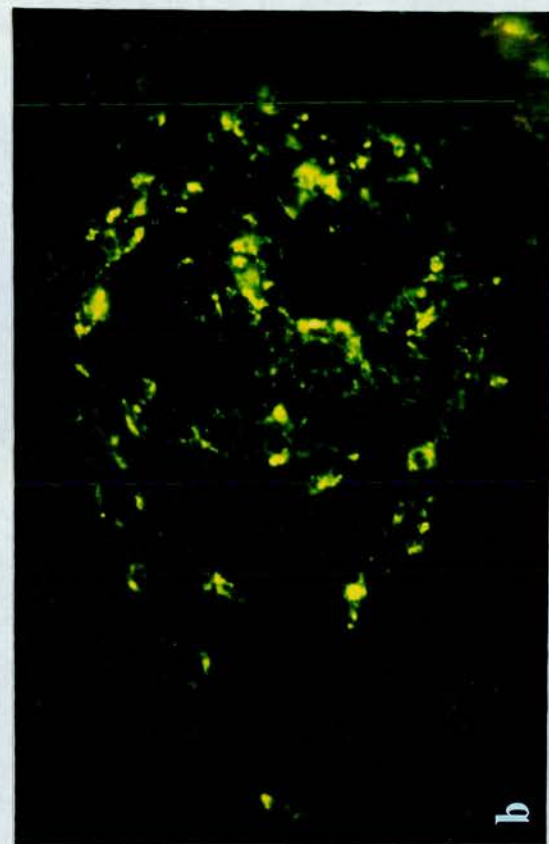


Fig 20 : DP BB/E rat pancreas at onset of
ketoacidosis (aged 115 days). X 312

(A) Double staining for glucagon (red)
and MHC class II positive cells.

(B) Double staining for glucagon (red)
and ED1⁺ cells (green).

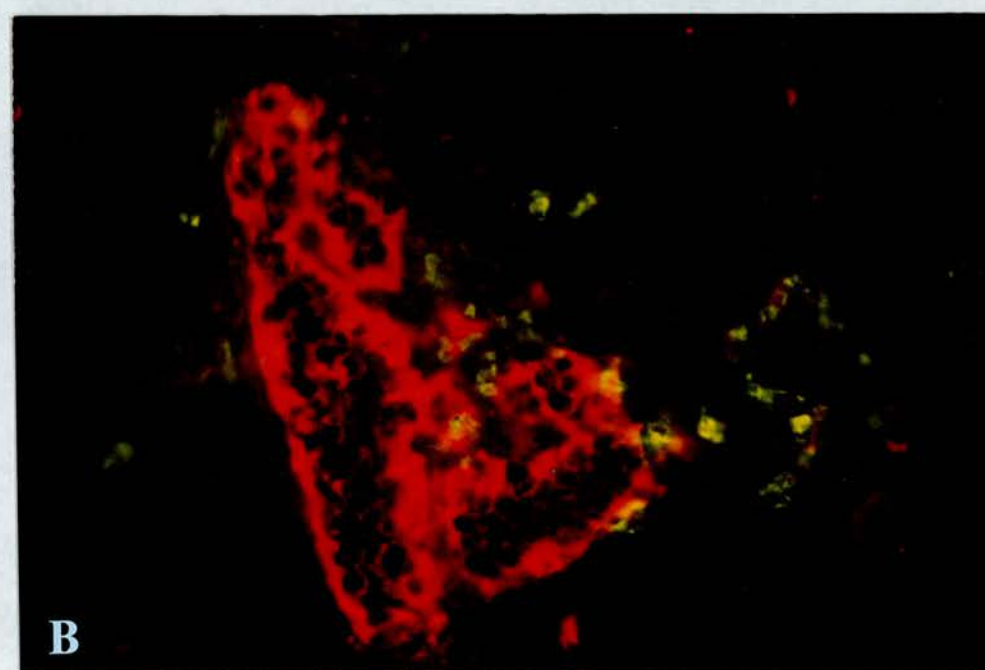
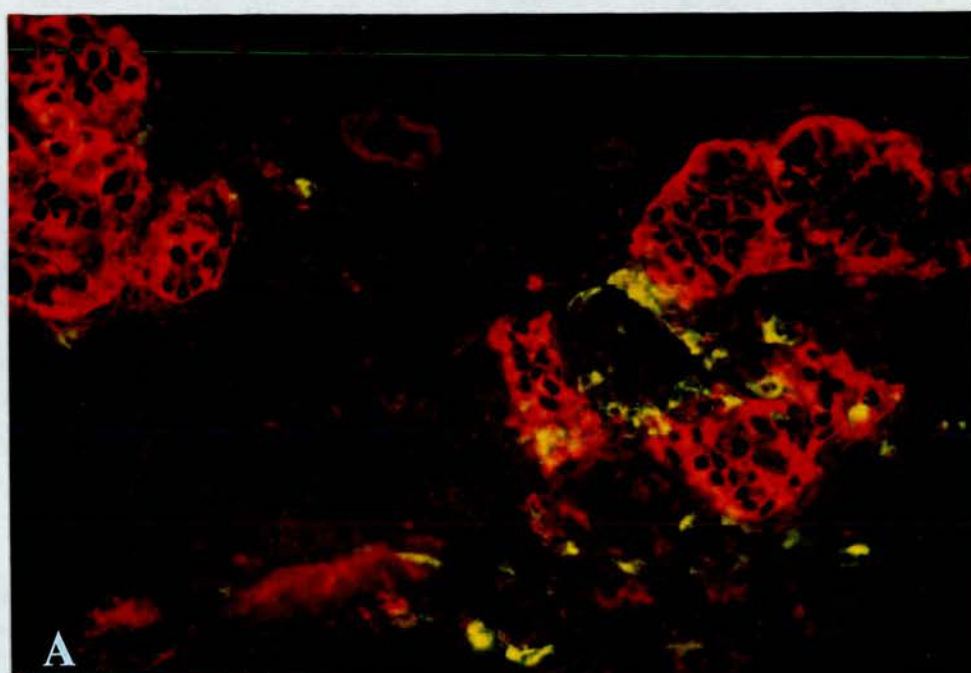
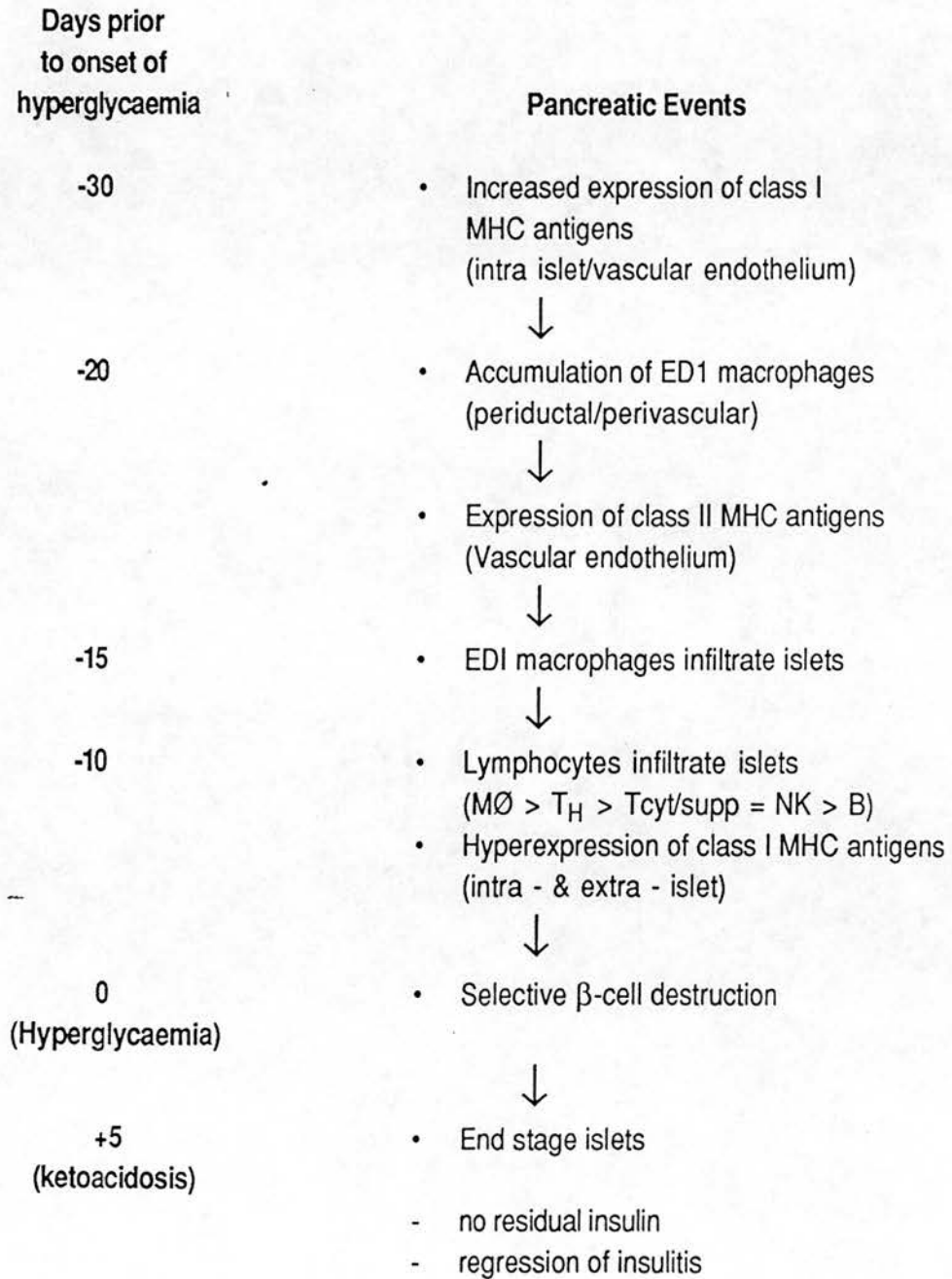


FIGURE 21: SCHEMATIC PRESENTATION OF THE SEQUENCE OF PANCREATIC EVENTS LEADING TO THE DEVELOPMENT OF IDDM IN THE BB/E RAT.



DISCUSSION

In 1983 Bottazzo et al (540), based on observations in thyroid tissue derived from patients with Graves' disease, postulated that aberrant expression of MHC class II antigens on target cells may permit presentation of cell specific autoantigens to potentially autoreactive T cells, thereby initiating an autoimmune response. Since many autoimmune diseases, including Type 1 diabetes, have HLA class II linked genetic susceptibilities (545), this was an attractive hypothesis linking the genetic susceptibility with an event on the target cell. However, the relationship of cause and effect between expression of class II MHC antigens and the development of autoimmunity has engendered much debate and controversy (546-548).

The finding of aberrant class II expression on some insulin containing cells in the fresh autopsy pancreas of a young child, with recent onset diabetes who died from ketoacidosis, provided some support to the hypothesis (261). However, in this single case, it was not possible to discern if the class II expression on beta cells preceded the development of insulinitis.

Evidence substantiating the above single case history, has recently been provided by the detailed studies of Foulis et al (549), who examined approximately 3000 islets in blocks of fixed pancreas obtained at autopsy from 35 diabetic patients who died from ketoacidosis.

Aberrant class II expression was found selectively on islet beta cells in 14% of insulin containing islets. However, whereas some islets were observed to contain class II expression on beta cells in the apparent absence of insulinitis, other islets were found with insulinitis but without aberrant class II expression. In pancreata examined from patients with recent onset

disease, 16% of the insulin containing islets displayed inappropriate class II expression on beta cells, of which 56% showed no evidence of insulinitis. It was stressed that insulinitis and aberrant class II expression could be quite focal within the islets and only by screening serial sections to study "whole" islets, could an accurate frequency of these phenomena be obtained. This was evident when "whole" islet analysis was performed on 130 islets from 3 patients with recent onset disease. Insulinitis was found in 44% of islets, where previously only 12% of islets had been found to be infiltrated when only a single section had been used for analysis on these patients. Similarly, aberrant expression of class II MHC molecules on islet beta cells was found in 67% of islets, compared with 26% on previous single section examination.

Analysis of "whole" islets was performed in my studies, by initially screening scan slides containing 8 sections/slide; each section having been taken at a different level through 80um of tissue. The results reported were based on the use of a mouse anti-rat MoAb directed against a nonpolymorphic determinant of the rat RT1B molecule. However, similar results were also obtained using another anti-rat class II MoAb, directed against the rat homologue of the mouse I-E molecule (RT1D).

The studies I have described in the BB/E rat, do not lend support to the proposal that aberrant expression of MHC class II molecules on pancreatic beta cells have a primary role in the process which ultimately leads to their destruction. Although occasional expression of MHC class II molecules, coincident with insulin containing cells, were observed in the first longitudinal cohort study, this occurred only in islets already heavily infiltrated. This suggests that, where

present, aberrant class II expression is a consequence, rather than a cause, of the disease process. It is possible that the aberrant class II expression may be induced by lymphokines such as interferon gamma released by activated T cells within the infiltrated islets (550, 551). However, in the subsequent and more detailed biopsy study, aberrant expression of class II MHC molecules on beta cells in islets, either with or without insulinitis, was not apparent. It is possible that the occasional MHC class II expressing beta cells observed in Study I may represent the phagocytic uptake of fragments of damaged insulin-containing cells by macrophages which are themselves expressing class II molecules (552). Indeed, electron microscopy and immunohistological studies on islets isolated from BB rats and streptozotocin treated rats at onset of diabetes, have revealed numerous class II positive islet cells that presented ultrastructural features of monocytes with vacuoles containing insulin immunoreactive granules (553). Equally in multiple low-dose streptozotocin-induced diabetes of mice, expression of MHC class II antigens was not found on endocrine cells but was restricted to phagocytic cells containing beta cell debris (554).

In a further study in the BB rat; Issa-Chergui et al (555) failed to detect class II MHC molecules on beta cells at the onset of disease. However, in this study, diabetes was induced in young BB rats by the passive transfer of ConA activated spleen cells from acutely diabetic BB rats and therefore the results might differ from the spontaneously occurring disease.

In the NOD mouse, conflicting results concerning the role of aberrant MHC class II expression, have been reported. Whereas Signore et al (556, 557) did not detect class II expression on endocrine cells, Hanafusa

et al (558) reported the expression of class II MHC antigens preferentially, but not specifically, on beta cells in 63% of islets of which 55% showed no evidence of insulinitis. However, class II expression was also found on islet cells from non diabetes prone BIO.GD and BALB/c mice. It is possible that cross reaction of the anti-class II MoAb with other tissue antigens, might have occurred. Equally, the use of Bouins fixed tissue and mouse anti-class II MoAbs in the latter studies, as opposed to cryostat sections and rat anti-class II MoAbs in the former studies, may contribute to the differences.

Recent studies which generated transgenic mice expressing MHC molecules on islet beta cells, aimed to determine if enhanced MHC expression on beta cells was in itself sufficient to lead to the development of IDDM (559-561). Major histocompatibility complex genes coding for the class I molecule H-2K^b (559) and the class II molecules, I-E^b (560) and I-A^d (561) were placed under the control of the insulin promoter. Strong expression of the transgenes were found in the targeted beta cells with only Lo et al (560) finding some expression also in the kidney tubule. In each case, IDDM ensued in the transgenic mice, but without the development of autoimmunity as determined by the complete absence of insulinitis either in mice syngeneic or allogeneic with the transgenes. Moreover, the effect on islet histology was variable. In the I-E transgenic animals, the islets remained intact. However, in the H-2K transgenics, fewer islets were observed, within which beta cell numbers were depleted and islet infrastructure was altered i.e. glucagon cells normally located at the periphery were intermingled with the remaining beta cells. In the I-A transgenics, immunoperoxidase staining for insulin showed reduced insulin staining in the transgenic mice.

Parham has suggested that IDDM in these transgenic animals results from the disruption of the pathway for biosynthesis, maturation and secretion of insulin (562). It is envisaged that active competition between insulin and class II molecules for factors in the intracytoplasmic compartments, may lead to the production of an inactive insulin. Alternatively, insulin secretion may be impaired by the binding of insulin to a peptide binding site on the class II molecule, similar to that described for the class I molecule (74).

Moreover, it was reported that expression of the H-2K^b and I-E^b transgenes rather than inducing an autoimmune response, resulted in the establishment of tolerance to the transgenes. In the study by Lo et al (560), T cells from transgenic mice gave strong proliferative responses in mixed lymphocyte cultures against allogeneic stimulators demonstrating their immunocompetence, but gave only weak responses to the I-E^b transgenic stimulator cells. Thus the T cells appeared tolerant to the I-E^b molecule despite the absence of I-E^b in the thymus or other lymphoid tissue. In addition, thymic lymphocytes from transgenic mice also responded poorly to the transgene product, suggesting that induction of tolerance to I-E occurs during T cell development in the thymus. Furthermore, Allison et al (559) reported the absence of T cells, even in mice allogeneic to the H-2K^b transgene, implying the development of tolerance to the transgene product. The authors suggested that shedding of transgenic molecules from the beta cell surface, may reach the thymus where they are reprocessed to induce tolerance. This is similar to the report by Adams et al (563) who studied transgenic mice expressing the SV40 T antigen, under the control of the insulin promoter. They found that expression of the transgene early in ontogeny induced tolerance to the SV40 T antigen.

Although these transgenic mice models of IDDM have not provided a valid test for the role of enhanced MHC expression in the development of Type 1 diabetes, they have provided interesting information on thymic education and mechanisms of tolerance. In this regard, Iwatani et al (564) have proposed that expression of class II MHC antigens on non lymphoid cells may ultimately induce tolerance against self antigens by protecting self from the immune response raised against nonself which might otherwise interact with self.

Although it was observations made in autoimmune thyroid disease which initially suggested a potential role for aberrant MHC class II expression in the initiation of autoimmunity (541), most of the subsequent studies in thyroid disease suggest that ectopic class II expression is not the primary event leading to the infiltration of autoreactive lymphocytes but rather is a consequence of that infiltration (565-567). Accordingly, the topographical distribution of ectopic class II expression on the follicular epithelium of autoimmune thyroid glands in vivo is particularly pronounced in the vicinity of the lymphoid infiltrates (568).

Similar findings to those I have described in the BB/E rat, have been reported for experimental autoimmune thyroiditis induced by neonatal thymectomy, where MHC class II positive thyroid follicular cells were found only in association with a T cell infiltrate in a few animals and was rare even in them (569). In the spontaneous thyroiditis of BB rats, thyroid epithelial cells remained negative for class II MHC determinants throughout the process of autosensitization (570).

In addition to autoimmune diseases, enhanced expression of class II antigens has also been reported on appropriate target cells during allograft rejection

(571) as well as many different organs during immune responses against antigens which are unrelated to the tissues themselves as in graft-versus-host disease (572-574). Moreover, although crypt epithelial cells in the gut are generally negative for MHC class II antigens, it is found that those cells adjacent to Peyer's patch follicles express class II molecules (575). These observations suggest that in many cases, the expression of MHC class II antigens on tissue cells is a normal physiological phenomena resulting from close proximity to an active immune system.

However, physiological expression of MHC class II molecules may also occur in the absence of immunological stimuli, as is found on mammary gland epithelium during lactation (576). Equally, class II MHC antigen expression has been reported on normal adrenal cortex tissue in the absence of lymphocytic infiltration, suggesting physiological expression of class II antigens may occur even in the absence of adjacent lymphocytes (577). Therefore, the finding in patients with IDDM of class II molecules on pancreatic beta cells in islets devoid of insulinitis (549), may not be "aberrant" but "physiological".

Pertinent to the hypothesis of aberrant expression of MHC class II antigens on non lymphoid cells being the trigger for autoimmunity, is the assumption that the mere acquisition of MHC class II molecules can bestow sufficient properties of a classical APC such as the macrophage (578) to permit the presentation of antigen. Several studies have reported the presentation of nominal antigens by a variety of non traditional class II positive accessory cells, including vascular endothelial cells (579), astrocytes (580) and fibroblasts (581). Cultured human thyroid epithelial cells with lymphokine induced class II expression, have

been shown able to present already processed viral antigen to antigen specific T cells (582). However, the presence of small numbers of classical APC's contaminating the cultures may have been responsible for the antigen presentation (583). The presence of contaminating cells among thyrocyte cultures, has been suggested by other studies. Whereas Pujol-Borrel et al (542) reported the induction of thyrocyte class II expression by the lectins PHA and ConA, subsequent studies have indicated that this lectin induced MHC class II expression is dependent on the presence of contaminating T cells within the cultures (567, 584). Furthermore, although murine thyroid follicular epithelial cells (TFEC) could be induced following culture with IFN-gamma to express MHC class II antigens and produce IL-1, the TFEC were consistently unable to present antigens either nominal (requiring processing) or alloantigens (not requiring processing) to a variety of sensitized T cells, T cell clones or hybridomas (585).

In Type 1 diabetes, a recent report by Markmann et al (586) addressed the antigen presenting function of MHC class II expressing beta cells by using transgenic mice that expressed a foreign class II (I-E) molecule on their islet beta cells. In vivo antigen presentation was assessed by the graft survival of foetal pancreas from I-E⁺ transgenic mice when transplanted into I-E⁻ recipients. Whereas I-E expressing beta cells could be recognized and rejected by immune effector cells if the response was initiated by conventional APCs following the injection of I-E⁺ spleen cells, class II positive beta cells were themselves unable to initiate an immune response by naive non tolerant T cells. In vitro studies using a T cell line specific for a peptide fragment of herpes simplex virus glycoprotein D (gD), indicated that although anti-gD specific T cells

proliferated when cultured with antigen and MHC compatible spleen cells, no proliferation of the T cells resulted from culture with antigen in the presence of I-E bearing islet cells. Thus, in both in vivo and in vitro experiments, I-E⁺ beta cells of transgenic mice were unable to present antigen to immunoreactive T cells.

Furthermore, it was found that rather than initiating an autoimmune response, antigen presented along with class II positive islet cells resulted in paralysis of antigen specific T cells, thereby inducing tolerance.

In concluding this part of the discussion, the studies in the BB/E rat and other animal models of IDDM, do not support the hypothesis that aberrant expression of class II molecules on beta cells is an essential event in the triggering of Type 1 diabetes. In the BB/E rat, expression of class II molecules was a rare phenomenon, which whenever found was always accompanied by insulinitis. Discrepancies between these studies and those in human IDDM, as reported by Foulis et al (549), where some islets were found with beta cell class II expression in the apparent absence of insulinitis, may reflect real species differences in the development of IDDM. However, they may also be accounted for by the fact that all the human pancreata studied, so far, have been obtained from patients already diabetic at which time it is difficult to define the sequence of earlier preceding events.

Presentation of nominal antigens has been described for a variety of class II bearing non lymphoid cells. However, there have been no reports demonstrating such antigen presentation capabilities of class II positive islet beta cells.

The steadily growing number of reports in the literature, documenting the induction of class II MHC molecules on an increasing variety of non lymphoid cells by cytokines such as IFN-gamma and their expression on many tissue cells following immune responses against unrelated antigens, suggests that such inappropriate class II expression may be more "physiological" than "aberrant". The purpose (if any) of this physiological class II expression is unclear, but it has been suggested that it may serve as an extrathymic mechanism for maintaining self tolerance (567, 586).

In their original report, Bottazzo et al (261), in addition to the aberrant expression of MHC class II antigens on beta cells, also described a marked increase in class I (HLA- A, B, C,) molecule expression in many islets irrespective of the presence of infiltration.

In the BB/E rat, using serial pancreatic biopsy to study sequential changes in individual animals, the first observed change was the increased expression of MHC class I molecules within some islets of animals aged 40 and 50 days, and therefore in the absence of insulitis, which in common with other BB rat colonies, has seldom been observed prior to 60 days of age (363, 587). Furthermore, enhanced MHC class I heavy chain gene expression has been demonstrated in pancreatic islets of 40 day old DP BB rats prior to the detection of low levels of complementary RNA for the TCR beta chain and IFN-gamma used as indicators of insulitis (588).

Leading up to the onset of IDDM, marked hyperexpression of class I molecules on both endocrine and non endocrine cells, in association with the presence of infiltration was observed, and this is similar to the findings of other groups (588, 589). At this stage in the disease process, the observed hyperexpression of class I

molecules is probably due to the release of cytokines such as IFN-gamma and TNF from the infiltrating immunocytes, where such cytokines are well known to be capable of upregulating MHC class I expression on a wide variety of cells (551, 590-592).

The observations in the BB/E rat, are supported by the findings of Foulis et al (549) in their studies of human IDDM using autopsy material derived from patients who died from diabetic ketoacidosis. Whilst recognizing the limitations imposed by cross-sectional data collected at a time when 80-90% of the beta cells have been destroyed, hyperexpression of class I MHC antigens on all endocrine cells was found in 92% of insulin containing islets, but in only 1% of insulin deficient islets. Although all insulin containing islets, affected by insulinitis, hyperexpressed class I MHC antigens, many of the islets in which hyperexpression of class I molecules was observed were without insulinitis.

The cause of the enhanced class I expression in the absence of insulinitis, is unknown. However, in a further study by Foulis et al (593), hyperexpression of class I MHC molecules was associated with the presence of immunoreactive interferon-alpha within insulin containing beta cells. It is possible that chronic viral infections, which can stimulate the release of interferons from epithelial cells which in turn have been shown to enhance the expression of class I MHC molecules on pancreatic endocrine cells (591), may be responsible for the upregulation of MHC class I antigens within islet beta cells. This is supported by the report of Campbell et al (594), demonstrating the enhancement of class I expression on both human beta cells and RINm5F cells following their infection with reovirus types 1 and 3.

A role for viral or bacterial infection in the pathogenesis of IDDM in the BB rat, had been discounted on the basis of an early study by Rossini et al (595) which raised animals under germ free conditions. Despite the gnotobiotic environment, the expected percentage of rats developed diabetes. However, this study does not exclude the possibility that vertically transmitted viruses, slow viruses or any agent that was not specifically tested for, may be involved in the aetiology of IDDM.

In human IDDM, evidence that viral infections are associated with the development of diabetes is derived from epidemiological studies, where evidence of Coxsackievirus B4 (596, 597) and rubella virus (598, 599) infections are common amongst IDDM patients. Yoon et al (600), reported that isolation of a strain of Coxsackievirus B4, from the pancreas of a child who died of ketoacidosis; when inoculated into certain strains of mice, induced diabetes. Furthermore, infection of mice with Coxsackievirus B4 has been shown to enhance the expression of the putative 64KD islet cell autoantigen on infected islets (601).

In rodents, diabetes following infection with encephalomyocarditis virus (602), Mengovirus (603) and Coxsackievirus B4 (604) is believed to result from direct cytolysis of the infected beta cells. In contrast, reovirus Type 1 (605) and rubella virus (606) infections can cause a mild transient form of diabetes, which can be prevented by immunosuppression (607) suggesting a role for autoimmunity in its development. However, whilst some viruses may be involved in the pathogenesis of some cases of IDDM, there remains no conclusive demonstration that IDDM is caused by viral infection.

The suggestion that some factor being produced within the beta cells themselves is responsible for the enhanced islet cell MHC class I expression, is compatible with the pancreatic morphology observed in both the BB/E rat and human IDDM studies. In the BB/E rat, the enhanced class I expression appeared to initially affect the beta cells at the islet core with subsequent spreading to affect all the endocrine cells within the islet. Since the order of the islet cellular perfusion and interaction is from the beta cell core, outward to the glucagon mantle (608), the observed class I expression on the glucagon cells would be in keeping with a possible paracrine effect caused by release of beta cell factors into the islet microvascular system. Furthermore, this is also suggested by the absence of increased class I expression in end stage islets where insulin containing beta cells and insulitis are no longer found.

The role of the enhanced islet cell class I expression in the pathogenesis of Type 1 diabetes, is unknown. In human IDDM, where cytotoxic T lymphocytes are reportedly the major infiltrating immunocyte (261), the upregulation of class I MHC proteins on islet cells may provide for their recognition and destruction by cytotoxic T cells. However, transplantation studies in the BB rat suggest that islet cell destruction is not MHC restricted (397). In addition, other studies have reported an absence of functional cytotoxic T cells in the BB rat (376, 389). In the BB/E rat, although some CD8 positive cells were detected amongst the infiltrating cells, they were not numerous. Furthermore, it is not possible to identify the CD8⁺ cells as T cyt cells, since this phenotype is also expressed by suppressor T cells. Accordingly, the enhanced class I expression may invoke the activation of a T supp cell population in an attempt to down regulate

autoaggressive immune responses (57, 58). It is also possible that the observed enhanced class I MHC expression has no pathological function but merely results as a consequence of perturbations within the islet microenvironment, where a decrease in beta cell volume in DP BB rats has been found to precede insulinitis (536). As such, enhanced class I expression within pancreatic islets, represents an early marker of impending immune assault on the islets.

Immune assault on the pancreatic islets of the BB/E rat as determined by insulinitis, was not observed in either of the longitudinal studies in any animal prior to 60 days of age. This appears to be a common finding amongst studies using BB rats from other colonies (312, 363, 536, 587). However, prior to insulinitis, pancreatic infiltration coincident with expression of MHC class II molecules on the ductular and vascular endothelium, is observed with cells accumulated around the pancreatic ducts and blood vessels.

The results from the pancreatic biopsy study, indicate that a population of macrophages identified by the MoAb, ED1 are prominent during this early phase of pancreatic infiltration. The studies revealed that ED1⁺ macrophages infiltrated the pancreas around the ducts and vessels and then migrated to the islets, approximately 2-3 weeks before the onset of IDDM. This is followed by infiltration of the islets by the ED1⁺ cells, thereby initiating insulinitis. This appeared to occur before the recruitment of other effector cells, since even by "whole" islet analysis, using serial sections, T- and B- lymphocytes were inconspicuous at this stage. Subsequent infiltration by T_H cells and to a lesser extent T cyt/supp cells, NK cells and B lymphocytes was only marked in biopsies taken 1-2 weeks before the onset of diabetes.

These results are similar to those recently reported by two other groups. The study by Hanenberg et al (589) used the same anti-rat macrophage MoAbs as employed in my studies, whereas Lee et al (587) used the MoAb OX-41 which recognizes granulocytes as well as macrophages (609). Equally, in low-dose streptozotocin-induced diabetes of mice (508), and in NOD mice (476, 610), a similar involvement for macrophages in the pathogenesis of IDDM has been reported. However, in the NOD mice studies, diabetes was induced by cyclophosphamide treatment, and thus may not reflect the spontaneous disease. In addition, no immunohistological studies identifying macrophages were performed and as such, the involvement of macrophages was inferred when insulinitis was prevented following the administration of silica, which is believed to selectively inhibit macrophage function (611).

Similar observations, to those found in the BB/E rat, have been reported for other autoimmune conditions. Induction of experimental autoimmune thyroiditis in the rat, following neonatal thymectomy, was associated with increased endothelial Ia expression and infiltration of the thyroid by ED1⁺ macrophages (569). De novo expression of class II MHC antigens, by ductular epithelium and vascular endothelium accompanied by perivascular infiltration by phagocytic mononuclear cells, is observed in streptozotocin-induced diabetes of mice (554). In the inflammatory central nervous system, lesions of Lewis rats with experimental allergic encephalomyelitis, ED1⁺ macrophages were prominent among the perivascular infiltrates (612).

Previous ultrastructural studies had suggested the involvement of macrophages in the development of IDDM in the BB rat (312). These studies were performed on diabetic animals at onset of disease and, therefore,

gave no indication of the prediabetic events. A further study, whereby administration of silica to BB rats prevented diabetes, also suggested a role for macrophages in the pathogenesis of IDDM (434). This study, however, did not provide histological data and therefore gave no indication as to where in the disease process, macrophages may be involved. As already mentioned, from the serial pancreatic biopsy study, it appeared that macrophages were the initial infiltrating cells causing insulitis. This is supported by the study of Hanenberg et al (589) where administration of silica was shown to prevent both the infiltration of ED1⁺ macrophages and subsequent T lymphocyte recruitment. Furthermore, in low-dose streptozotocin-induced diabetes, in vivo administration of MoAbs, against T lymphocyte subsets, prevented the islet infiltration by T cells but not by macrophages (508). Thus, the evidence suggests that macrophage infiltration precedes lymphocytic infiltration and that lymphocyte recruitment is dependent upon the presence of macrophages.

However, although macrophages appear involved in the early stages of the disease process, the studies in the BB rat (369), the NOD mouse (476) or in low-dose streptozotocin-induced diabetes (508), which show that in vivo depletion of T cell subsets can ameliorate the disease, indicates that T cells are also necessary for the manifestation of the disease.

Although it is not known what activates the immune response against the pancreatic islets, the immune response involves the diapedesis of lymphoid elements from the circulation to the pancreatic islets. Accordingly, inhibition of vascular permeability has been shown to prevent low-dose streptozotocin-induced diabetes (514). Recently, Majno et al (613) found evidence of a specific pancreatic venular defect in the

BB rat. Ultrastructural studies suggested that a population of marginating intravascular monocytes, when activated, may induce venular leakage through the release of IL-1 (614), and hence allow enhanced diapedesis of lymphoid cells.

It is possible that this population of intravascular monocytes may correspond to ED1⁺ cells since ED1, but not ED2, stains the majority of peripheral blood monocytes (529), and ED1⁺ cells have been seen trapped in the vascular endothelium of Wistar rats (615).

Following their accumulation at perivascular sites, the ED1⁺ macrophages are attracted to the pancreatic islets and although the agent responsible for this has not been identified, Leiter (616) has shown that insulin itself can be chemotactic for activated macrophages. Having infiltrated the islets, ED1⁺ cells may release cytokines such as IL-1 (411) and TNF (412) which not only affect insulin secretion but also can be cytotoxic to islet beta cells. Furthermore, release of such cytokines can lead to the generation of a self-perpetuating inflammatory response. Accordingly, IL-1 (617, 618), TNF (619, 620) and IFN-gamma (620, 621) can activate endothelial cells, of pancreatic vessels and ducts, to bind increased numbers of lymphocytes. Subsequent migration of lymphocytes, through the endothelial cell layer, is enhanced by release of IFN-gamma (622). The infiltrating lymphocytes may then be attracted to islets already infiltrated by macrophages by an IL-1 chemotactic gradient (618, 623).

Lymphocyte emigration may also be influenced by the expression of MHC class II molecules on the vascular and ductular endothelium and on islet capillary endothelial cells, whereby endothelial cell class II expression could be used to recruit antigen-specific T_H cells into

the site of a developing immune response, serving as an amplification mechanism (624). As such, MHC class II expression, on endothelial cells, can favour homing of lymphocytes to particular tissues in autoimmune diseases as has been shown for rheumatoid arthritis (625).

In lymphoid tissue, emigration from the circulation of the mononuclear cells which participate in immune responses, occurs in specialized post capillary venules known as high endothelial venules (HEV)(626). High endothelial venules can also develop in chronically inflamed tissues (627) and recently, Kabel et al (628) described the late development of HEV, containing intrathyroid lymphoid tissue in BB rats with spontaneous autoimmune thyroid disease. Similar HEV might be expected to develop in the pancreas of BB rats, thereby improving the homing and recruitment of lymphocytes to the sites of inflammation.

Furthermore, Jalkanen et al (625) found that some B cell clones, that adhered to HEV of lymph node and mucosa-associated lymphoid tissue (MALT), were unable to bind to HEV in rheumatoid synovium. In addition, use of the MoAb MEL-14, which blocks lymphocyte adherence to MALT-HEV, did not inhibit the binding of lymphocytes to synovial HEV. This suggested that there may be a novel class of homing receptors on certain subpopulations of lymphocytes distinct from the receptors for the "classical" HEV of secondary lymphoid tissues, thereby enabling their specific homing to the synovium. It would be speculative to suggest that similar lymphocyte homing receptors, specific for possible pancreatic HEV, might exist.

Cytokines produced in the inflammatory response e.g. IL-1, TNF and IFN-gamma, can also induce certain intercellular adhesion molecules (ICAM) such as ICAM-1

on a variety of cell types including endothelial, epithelial and myeloid cells (629). ICAM-1 is the ligand for the antigen LFA-1 (lymphocyte function associated) which is present on all leucocytes and is believed to strengthen the adhesion between leucocytes and their target cells (630). Thus, within infiltrated islets, local cytokine production may induce ICAM-1 expression on islet beta cells, thereby enhancing specific leucocyte-beta cell interactions. Recently, at the 7th International Congress of Immunology, Campbell et al (631) reported the in vitro induction of ICAM-1 expression by TNF and IFN-gamma, on islet beta cells. The ICAM-1 expression was however also induced on the other islet endocrine cells and therefore does not reflect the beta cell specificity of IDDM. However, in vivo, it may be that islet ICAM-1 expression is more selectively distributed.

Although both macrophages and T cells appear necessary for the development of IDDM in the BB rat, phenotypic analysis, such as I have performed in the BB/E rat, does not allow the elucidation of their functional roles. In biopsies taken 1-2 weeks before the onset of IDDM, infiltrated islets contained a variety of different cell types. Whereas ED1⁺ macrophages were the most numerous cell population within the infiltrated islets, this does not necessarily imply they are the most important. Accordingly, it is not known which of the infiltrating cells ultimately deliver the lethal-hit to the beta cells and which cells, "like the crowd gathered at the scene of an accident", merely represent innocent bystanders.

Macrophages infiltrating the islets may directly damage the pancreatic beta cells, perhaps via release of beta cell cytotoxic cytokines (411, 412), and/or may act as classical APCs and present beta cell specific antigens

to MHC class II restricted T_H cells which infiltrate the islets. Evidence for macrophage mediated cytotoxicity towards cultured pancreatic islets has been reported (632). In addition, Nagy et al (633) recently demonstrated that splenic macrophages isolated from diabetic BB rats, exhibited enhanced killing of islet cells isolated from DR BB rats. Furthermore, they suggested that the potential for splenic macrophage mediated cytotoxicity develops prior to insulinitis, and that trafficking of macrophages from the spleen to the islets may occur.

Amongst the infiltrating T cell population, T_H cells were the predominant phenotype in the BB/E rat, and this has similarly been reported for the NOD mouse (557) and low-dose streptozotocin-induced diabetes (508). Activation of the T_H cells, following presentation of antigen by MHC compatible macrophages within the pancreatic islets may serve to initiate activation and recruitment of other effector cells. Alternatively, some of the $CD4^+$ cells, may directly exert a cytolytic effect on the beta cells (634, 635). Equally, NK cells, which are also present amongst the infiltrating cells, may mediate beta cell destruction as has been suggested by other studies (367-369).

The lytic process of beta cell destruction is not fully understood, but recent studies based on the Okamoto model, for pancreatic beta cell damage (493), suggest an involvement of free radicals in the mediation of islet cell damage (636). The Okamoto model views the generation of free radicals such as superoxide anions and hydroxyl radicals produced in the inflammatory response, as principal mediators of islet tissue damage. In addition to attack on the cellular membrane, these radicals can induce strand breaks in the islet cell DNA (490). DNA strand breakage promotes activation of the

enzyme poly-ADP-ribose synthetase to initiate DNA repair. This consequently depletes the islet NAD content, thereby inhibiting proinsulin synthesis and increasing islet sensitivity to further free radical damage (493).

Support for the model comes from studies whereby inhibition of the formation of these free radicals can prevent islet cell damage. Combined treatment with the iron chelator desferrioxamine, and the poly-ADP-ribose synthetase inhibitor nicotinamide, has been shown to prolong islet allograft survival in diabetic NOD mice (471), and in multiple low-dose-streptozotocin-induced diabetes (489). Interestingly, in the NOD mouse, treatment with dimethyl sulphoxide (DMSO), a hydroxyl radical scavenger, contrary to expectations, increased the rate and frequency of diabetes whereas its in vivo metabolite, dimethyl sulphide (DMS) reduced the incidence and rate of diabetes (637).

In the BB rat, a time-course study was performed to evaluate the intra-islet activity of the superoxide radical scavenger; superoxide dismutase in relation to the development of IDDM (638). Although SOD activity did not alter with age or the development of diabetes, BB rats were found to have a lower endogenous SOD activity than Wistar control rats. Thus, the lower SOD activity may be a susceptibility factor that favours the development of IDDM in the BB rat.

Accordingly, a high susceptibility for oxygen radicals by beta cells compared with the other islet cells, due to a low radical scavenger potential by the insulin producing cells, has been suggested to account for the specificity of beta cell destruction in IDDM (639).

In conclusion, whilst the precise mechanism of beta cell destruction is still unclear, the use of serial pancreatic biopsy has enabled documentation of the following sequence of events in the development of IDDM in the BB/E rat.

The first phenomenon observed, is enhanced expression of MHC class I molecules on islet cells. The cause of this is unknown, but chronic viral infection remains a possibility. This is followed by emigration from the circulation, by a population of macrophages which accumulate adjacent to pancreatic ducts and blood vessels expressing MHC class II molecules on their endothelium. Migration of these macrophages, into the pancreatic islets, soon follows thereby initiating insulinitis. Release of cytokines from the activated macrophages may directly injure the pancreatic beta cells, but probably initiates a cascade of events including the induction of adhesion molecules and homing receptors and increasing vascular permeability, all of which aid and abet the subsequent recruitment of T- and B- lymphocytes into the inflammatory lesions. Within the infiltrated islets, the invading immunocytes either individually or collectively serve to selectively destroy the islet beta cells.

The progressive spreading of these pathological phenomena, with resultant beta cell loss, will eventually result in the onset of IDDM when 80-90% of the beta cell mass has been destroyed, at which point administration of exogenous insulin is required to sustain life.

**CHAPTER 7 - CYTOKINE MODULATION OF MHC EXPRESSION ON
RAT PANCREATIC ISLETS:IN VITRO STUDIES**

INTRODUCTION

Cytokines are a group of soluble, small molecular weight (<80KD), antigen non-specific, protein cell regulators, collectively including lymphokines, monokines, interleukins and interferons (640). They are synthesized and secreted by a wide variety of cells in picomolar concentrations where they play an important role in regulating the amplitude and duration of both physiological and pathological immune responses. Individual cytokines can exert multiple effects on a variety of cells and thus, overlap in their biological effects on lymphoid, myeloid and connective tissue cells (641, 642). Consequently, a network of cytokine interactions exists, whereby cytokines can induce each other; modulate their cell surface receptors; and exert synergistic or antagonistic functions on their target cells (640).

Interferon-gamma, a lymphokine produced by activated T lymphocytes (642, 643), can enhance expression of MHC class I molecules and induce MHC class II molecules on a variety of cell types (644, 645). Interferon-gamma has been shown to enhance MHC class I expression and induce class II antigen expression on both rat (646) and human (551) thyroid cell cultures. However, whilst IFN-gamma has unequivocally been found to enhance MHC class I proteins on isolated mouse (647), rat (648) and human (591, 649) pancreatic islet cells, there are conflicting reports regarding the induction of class II molecules on isolated islet cells. Whereas some studies have failed to induce MHC class II molecules (591, 647, 649), other studies have reported the induction of class II antigens on dispersed mouse (650) and human (651) islet parenchymal cells, and on isolated rat islets (648).

However, induction of MHC class II molecules on islet cells by IFN-gamma was limited to 10-40% of the mouse islet cells (650) and 15-20% of the human islet cells (651). The study by Markmann et al (648) did not provide any quantitative data, but the authors emphasized that "not all treated islets in a given section nor all cells within a single islet were ever found to be highly positive for Ia". Furthermore, dual immunofluorescence studies revealed that the induction of MHC class II molecules included both beta and non-beta cells (650).

Further studies have shown that where IFN-gamma alone was unable to induce MHC class II molecules on monolayer cultures of human pancreas (652), or isolated human and mouse pancreatic islets cells (592); when combined with tumour necrosis factor (653), induction of class II MHC proteins on 30-50% of the islet beta cells was observed. However, in addition to the islet beta cells, class II expression was also found on the islet glucagon- and somatostatin-containing cells (652).

It is possible that the propensity to induce MHC class II molecules by IFN-gamma on pancreatic beta cells may depend on genetic influences, and this may account for some of the discrepancies between the above mentioned studies. Accordingly, the in vivo susceptibility of mice to the cytotoxic action of streptozotocin on islet beta cells is MHC related (500), and the levels of cytokine production may be related to MHC genotype (654).

Following the in vivo observations, concerning pancreatic MHC class II expression in the BB/E rat; which I have described in the previous chapter, it was important to investigate the role of cytokines on the modulation of both MHC class I and class II molecules on

pancreatic islet cells isolated from BB/E rats. In particular, we investigated the possibility that islet cells isolated from autoimmune, diabetes-prone animals, may be more susceptible to the induction of MHC class II molecules than islets isolated from non-diabetes-prone animals.

MATERIALS AND METHODS

ISLET ISOLATION, CULTURE AND DISPERSION

Islets were isolated by collagenase digestion as described in Chapter 4, from the following groups of animals: (i) ICSA positive DP BB/E rats aged 80-90 days; (ii) ICSA negative DP BB/E rats aged 80-90 days; (iii) DP BB/E rats aged 30 days; (iv) DR BB/E rats aged 80-90 days; (v) DR BB/E rats aged 30 days; (vi) normal Wistar rats aged 80-90 days; (vii) normal Wistar rats aged 30 days.

For each experiment, isolated islets were pooled from 4 rats in each animal group. Batches of pooled islets (350-400) were precultured for 2-3 days in tissue culture medium (RPMI 1640 containing 11.1mmol/l glucose; 20mmol/l HEPES; 100U/ml penicillin; 0.1mg/ml streptomycin and 10% heat inactivated foetal bovine serum). Islets were further maintained in free-floating culture ($37^{\circ}\text{C} + 5\% \text{CO}_2$) for 3-10 days in tissue culture medium with or without the addition of the following cytokines, either singly or in combination: (i) recombinant (r) rat interferon-gamma (r.IFN-gamma; 10^{-10^3}U/ml); (ii) human or murine recombinant tumour necrosis factor (r.TNF; 10^{-10^5}U/ml); (iii) murine recombinant interleukin 1 (r.IL-1; 5^{-10^3}U/ml). The fully supplemented culture medium was changed every three days. At the end of the culture period, single cell suspensions from the cultured islet cells were prepared using an enzyme dispersion technique (655).

The islets were washed twice in sterile 15ml Falcon tubes containing 6ml of ice cold Ca^{2+} and Mg^{2+} -free Hanks' balanced salt solution (pH 7.4) containing penicillin and streptomycin (as above) and BSA (5mg/ml)(HBSS- $\text{Ca}^{2+}/\text{Mg}^{2+}$). The washed islets were resuspended in 5ml of HBSS- $\text{Ca}^{2+}/\text{Mg}^{2+}$ containing the

following additions: 3mg/ml collagenase; 2.5mg/ml trypsin (Difco Laboratories, Detroit, MI); 1mg/ml hyaluronidase (Sigma, Poole, Dorset); 1.0µg/ml DNase (Sigma) and 1mmol/l EGTA (Sigma) and incubated for 20 mins. at 37°C in a shaking water bath to disperse the islet cells. Cell dispersion was completed by repeated (10 times) aspiration in a Pasteur pipette. The cell suspension was washed by repeated centrifugation (1 min. at 100g); twice in HBSS-Ca²⁺/Mg²⁺ and once in RPMI 1640 (supplemented as above). Cell numbers and viability were estimated by acridine orange/ethidium bromide (0.25µg/ml; 5µg/ml)(Sigma) staining of cell preparations on a haemocytometer. Cell viability exceeded 90% and islet cell suspensions containing 5x10⁵ cells/ml were finally resuspended in RPMI 1640 for subsequent immunofluorescence detection of rat MHC antigens.

ISLET CELL IMMUNOFLUORESCENCE STAINING

The MoAbs (Table I) MRC OX-6 and MRC OX-18 were used to detect rat MHC class II and class I antigens respectively. MRC OX-8 and MRC OX-19 were used as controls for non specific reactivity, and ED1 and ED2 used to detect potential contaminating cells of monocytic origin.

The MoAbs were made up in PBS (0.05mol/l, pH7.6) containing 10% normal rat serum, 1% BSA and L-lysine (20mmol/l)(Sigma) and used at 1:2-1:5 dilution for the clone supernatants, and 1:100-1:300 for the ascites preparations. 100µl of dispersed islet cell suspensions (2x10⁵ cells/ml) were incubated with the diluted MoAbs (100µl) at 0-4°C for 1hr. Following three washes with 0.2ml PBS containing 1% BSA and L-lysine (20mmol/l), surface staining was revealed after a similar incubation with 0.1ml rhodaminated rabbit anti-mouse immunoglobulins (Dakopatts, Weybridge, Surrey, UK),

previously adsorbed with rat liver acetone powder (10mg/ml)(Sigma) and diluted 1:20 in PBS+BSA/L-lysine. After three washes (0.2ml PBS+BSA/L-lysine), the surface-stained cells were fixed in 0.1ml ice-cold 2% paraformaldehyde in 0.9% NaCl containing HEPES (20mmol/l) and BSA (1mg/ml) pH7.4 for 30 mins. The cell suspensions were washed three times in 0.2ml PBS containing 1% BSA, L-lysine (20mmol/l) and 2% foetal bovine serum and finally resuspended in 50µl of PBS.

The surface-stained cells were put onto PTFE coated glass slides (4 multispot)(C.A. Hendley, Essex, UK) and allowed to air dry. The slides were placed in ice-chilled acetone for 15 mins, air-dried and washed in PBS.

Subsequent double immunofluorescence staining to identify endocrine hormones was performed by incubating the slides for 1hr at 25°C with the following antisera: clone supernatants of monoclonal anti-insulin (2F1), anti-glucagon (GLU001) or anti-somatostatin (SOM018) and the polyclonal antisera M1183 and M1187 (guinea pig antibodies against insulin) were kindly provided by Novo, Copenhagen, Denmark. Further polyclonal antisera against insulin and glucagon hormones were obtained from Atlantic Antibodies (Winnersh, Berks, UK). Reactions of MoAb binding were revealed using biotinylated horse anti-mouse immunoglobulins (Sera Lab, Crawley Down, Sussex, UK) diluted 1:50 in PBS+BSA/L-lysine containing 10% normal horse serum (Sera-Lab), incubated for 40 mins., followed by fluoresceinated streptavidin (Cambridge Bioscience, Cambridge, UK) diluted 1:100 in PBS+BSA/L-lysine and incubated for 30 mins. Polyclonal antisera were identified with fluoresceinated antibodies to guinea pig or rabbit immunoglobulins (Cambridge Bioscience) diluted 1:20-1:40 in PBS+BSA/L-lysine

containing 10% nonimmune host derived serum and incubated for 30 mins. at 25°C.

A rabbit polyclonal antibody specific for factor VIII related antigen (Cambridge Bioscience) was used as a control for non-specific binding. Further controls, included primary incubations with PBS+BSA/L-lysine, followed by the fluoresceinated or rhodaminated conjugates, or primary incubations with normal mouse, rat, sheep, goat, guinea pig or horse serum (Sera-Labs) followed by the labelled conjugates.

Slides were examined under a Leitz Orthoplan microscope fitted with a fluorescence vertical illuminator and filter blocks for revealing fluorescein and rhodamine fluorochromes. Photographs were taken on Ektachrome ASA 400 film with automatic exposure.

CYTOKINE PREPARATIONS

Rat recombinant interferon-gamma (4×10^6 U/mg protein) was a generous gift from Dr Peter van der Meide (The Primate Centre, TNO, Rijswijk, The Netherlands). Its production in chinese hamster ovarian cells transfected with a chromosomal rat IFN-gamma gene, and its purification by antibody affinity chromatography have previously been described (656, 657).

Recombinant human tumour necrosis factor (2×10^8 U/mg) produced by Genentech, San Francisco, CA, was a gift from Dr G.R. Adolf (Boehringer-Ingelheim, Vienna, Austria). Recombinant murine tumour necrosis factor produced in E.Coli containing an appropriate plasmid, and purified to homogeneity (658) was kindly donated by Dr Jan Tavernier (Biogent, Geneva, Switzerland). The specific activity, as measured in a cytotoxicity assay on L929 cells, was 2×10^8 U/ml.

Recombinant murine interleukin-1 (10^4 U/ml) was a kind gift from Dr Peter Lomedico (Hoffman-La Roche, Nutley, NJ).

RESULTS

The results to be described, represent consistent findings using batches of pooled islets from experiments involving four animals per group. Each experiment has been reported on at least four separate occasions.

INTERFERON-GAMMA

Enhancement of MHC class I antigens on RINm5F cells by IFN-gamma has been well documented (592, 659-662). Therefore, in each set of experiments, cultures of RINm5F cells with or without added IFN-gamma were stained for class I MHC molecules to provide a positive control for the biological activity of the IFN-gamma preparation.

Figure 22 indicates the enhancement of MHC class I antigens on RINm5F cells after 3 days culture with 100U/ml r.IFN-gamma. This concentration of r.IFN-gamma had previously been established as optimal for Ia induction on cultured rat thyroid cells (646). Culture of islets with r.IFN-gamma (100U/ml for 3-10 days) increased the expression of MHC class I molecules on the surface of beta and non-beta islet cells, isolated from all the groups of animals studied (Fig. 23). The level of enhanced class I expression was not uniform, with some cells showing more intensive staining than others (Fig. 23a, Fig. 23c).

Islets isolated from the various animal groups and cultured with rat r.IFN-gamma were examined for the presence of class II MHC proteins. After 4 days in culture with r.IFN-gamma (100U/ml), islets isolated from both ICSEA-positive and ICSEA-negative DP BB/E rats, aged 80-90 days, were found to express MHC class II molecules (Fig. 24b). The islet cell suspensions prepared by

enzyme dispersion contain predominantly (>80%) beta cells (413), and approximately 50% of the insulin-containing cells were positive for class II antigens. The induction of class II antigens, although not present on all beta cells, was nevertheless specific for insulin-containing cells, since class II antigens were not detected on any glucagon-or somatostatin-containing cells from the same islet cell suspensions (Fig. 24d). Further-more, MHC class II molecules were not observed on any endocrine cells isolated from age-matched DR BB/E rats, normal Wistar rats or young (30 days of age) DP BB/E rats.

A small population (3-5%) of cells bearing MHC class II antigens were identified in cell suspensions from all groups of animals cultured with r.IFN-gamma (Fig. 25a). This probably reflects in part, some non-specific binding since reactions with the irrelevant antibodies OX-8 and OX-19 identified approximately 2% of the cells in all the cultures irrespective of the presence or absence of r.IFN-gamma (Fig. 25b). Equally, some of the non-endocrine class II expression may represent induction by r.IFN-gamma on surviving contaminating pancreatic ductal or exocrine cells (591). However, contamination of the cultures with macrophage-like cells was minimal, since reactivity of the cell suspensions to the MoAbs ED1 and ED2 were only comparable with the 1-2% non-specific binding of other irrelevant antibodies.

TUMOUR NECROSIS FACTOR AND INTERLEUKIN-1

The failure to induce class II MHC antigens on islet cells isolated from young DP BB/E rats suggested the possibility that islets isolated from the older DP BB/E animals may be susceptible to induction of class II molecules by r.IFN-gamma because of previous cytokine-mediated damage in vivo, prior to their isolation.

Thus, islets isolated from DR BB/E rats and normal Wistar rats, both aged 30 days, were cultured with r.TNF (both human and murine) or r.IL-1 either singly or in combination with r.IFN-gamma and examined for expression of MHC antigens.

Islets from both groups of animals, when cultured with r.TNF (10U/ml) alone for 3 days, showed enhanced levels of MHC class I expression (Fig. 26b), which was further augmented when combined with 100U/ml r.IFN-gamma (Fig. 26c).

Induction of MHC class II antigens on islet endocrine cells were not observed in either of the animal groups following incubation with r.TNF alone ($10-10^5$ U/ml) or in combination with r.IFN-gamma (100U/ml) over a 3-10 day culture period. Incubation of islets with r.IL-1 (5-1000U/ml) did not enhance class I expression, nor when combined with r.IFN-gamma (100U/ml) induce MHC class II proteins on islet endocrine cells.

Although these investigations were aimed to study cytokine modulation of MHC expression, it was observed that the numbers of islet cells recovered from the cultures containing r.TNF or r.IL-1 with or without r.IFN-gamma appeared decreased. Accordingly, it was found that r.TNF (>50U/ml) or r.IL-1 (>10U/ml) exerted cytotoxic effects on islets isolated from both DR BB/E rats and normal Wistar rats. After 4-5 days of culture, in the presence of either r.TNF or r.IL-1, marked morphological disruption of islet plasma membranes were observable under the stereomicroscope (Fig. 27).

Fig 22 : Expression of MHC class I proteins on
RIN m5F cells after 3 days culture in the
absence (A) or presence (B) of 100U/ml of
rat r.IFN-gamma. X 500

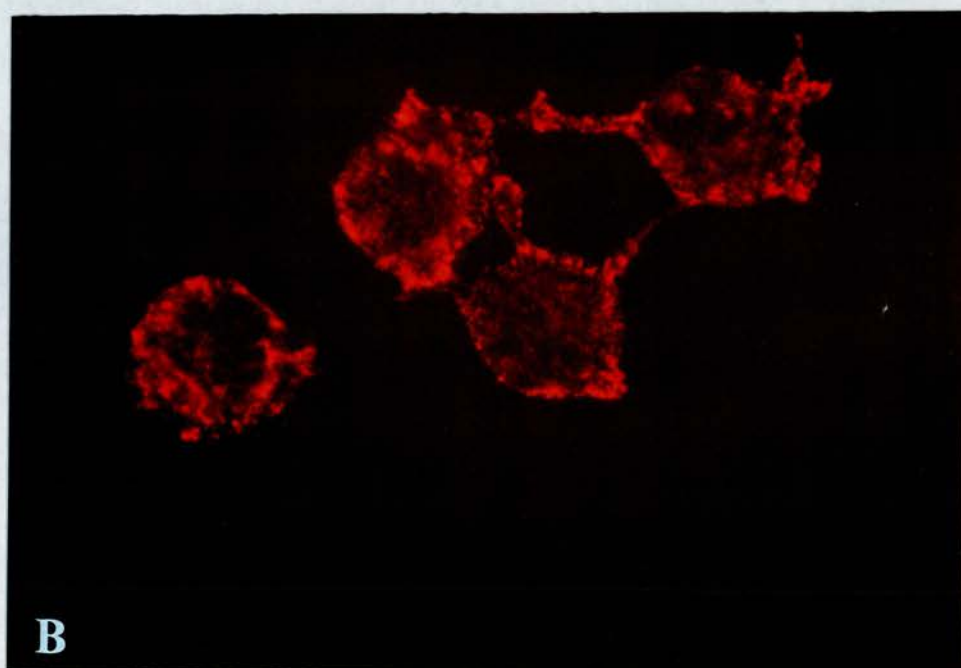
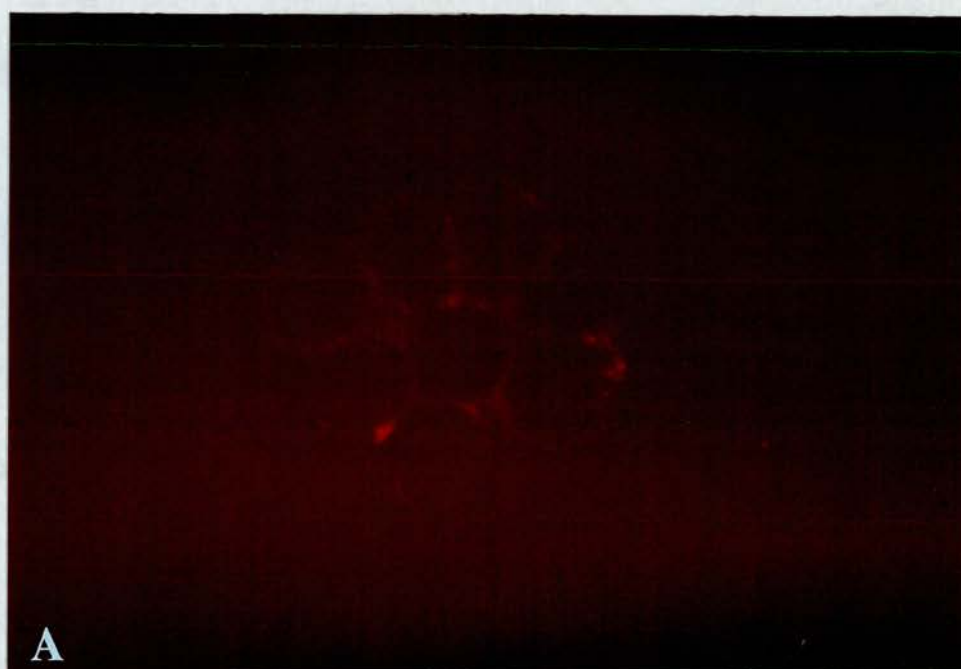


Fig 23 : MHC class I antigen expression on dispersed islet cell suspension from islets cultured with 100U/ml of r. IFN-gamma for 4 days.

- (a) OX-18 staining of islet cells from DR BB/E rats (80-90 days of age). X 312
- (b) double exposure for insulin-containing cells (green) and MHC class I positive cells.
- (c) OX-18 staining of normal Wistar rat islet cells (80-90 days of age). X 500.
- (d) double exposure for insulin (green) and class I MHC antigens (red).

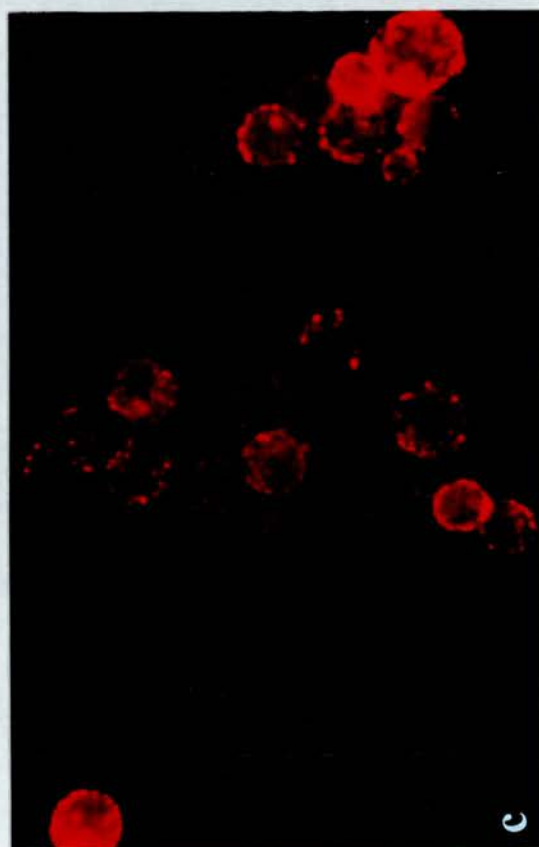
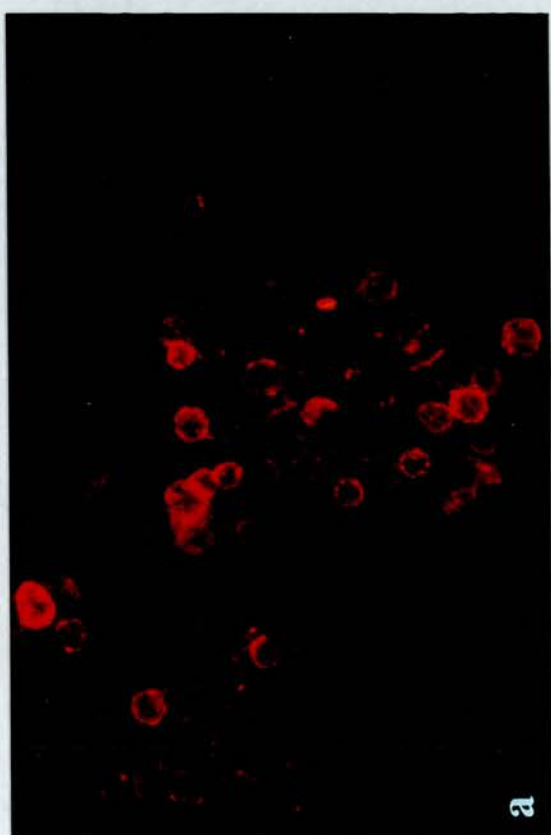
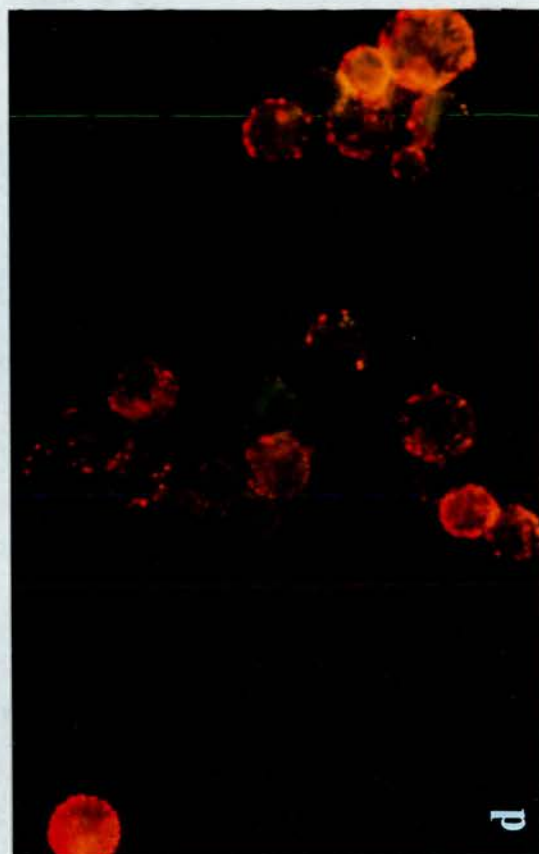
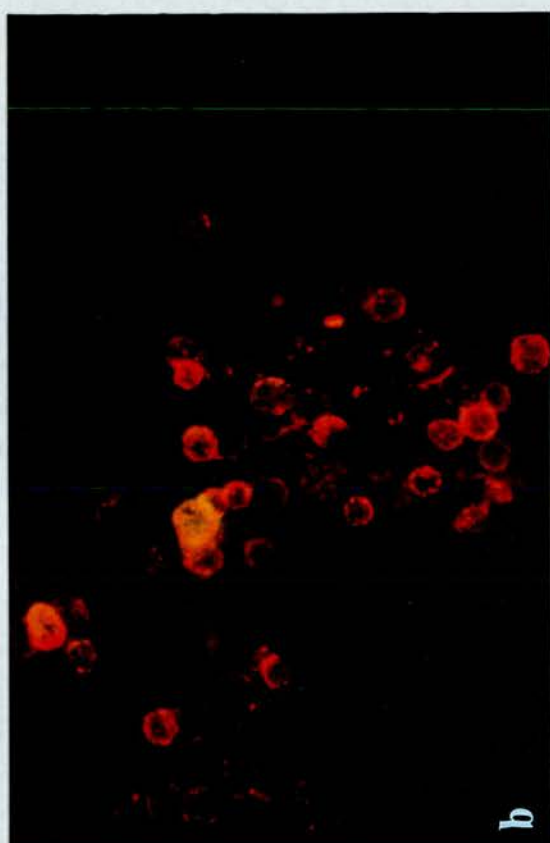


Fig 24 : Dispersed islet cell suspensions from isolated islets of ICSEA-positive DP BB/E rats (aged 80-90 days) cultured with 100U/ml of r. IFN-gamma for 4 days. X 500.

- (a) OX-6 staining for MHC class II positive cells.
- (b) double exposure of (a) for insulin (green) and OX-6 (red) shows the induction of MHC class II antigens on the insulin-containing cells in this field.
- (c) glucagon/somatostatin staining within the same cell suspensions.
- (d) double staining of the cells in (c) for class II MHC antigens shows they remain Ia negative.

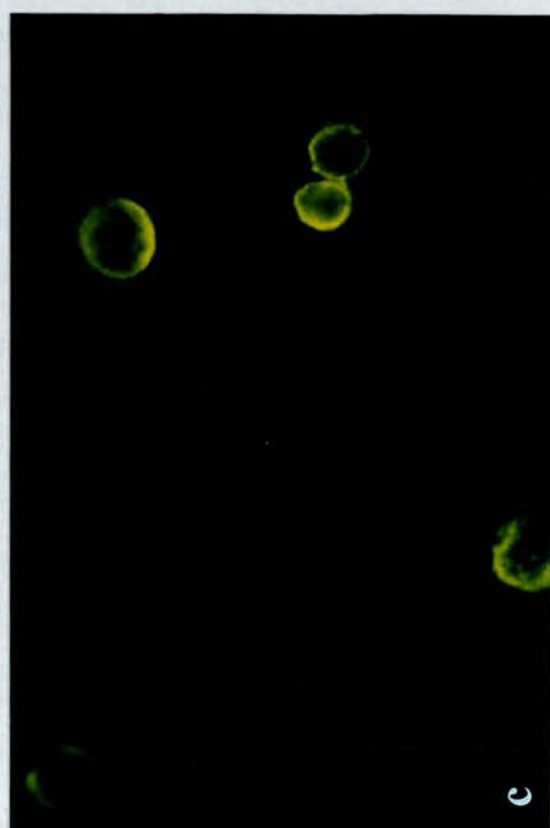
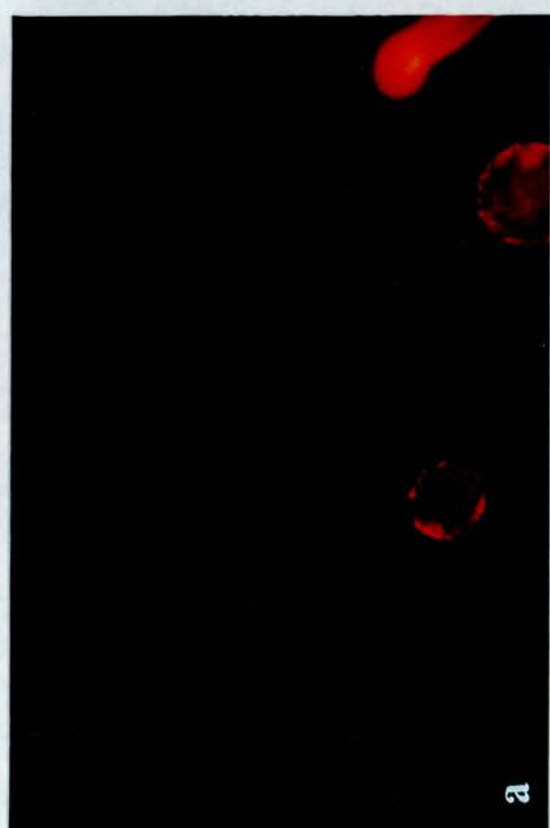
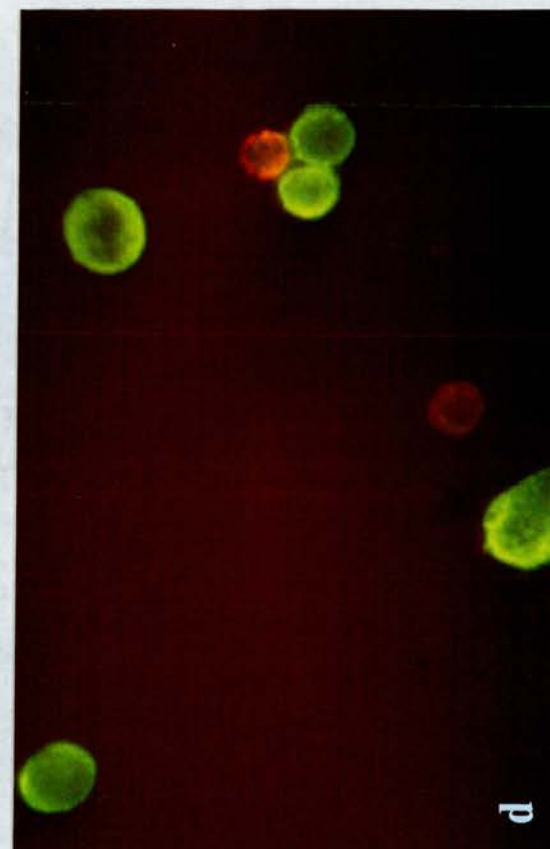


Fig 25 : Dispersed islet cell suspensions from isolated islets of 30 day old DR BB/E rats.

- (A) Double staining for insulin (green) and OX-6 (red) in cell suspensions cultured for 4 days with 100U/ml r.IFN-gamma shows some non-endocrine class II positive cells. X 500
- (B) Double staining for glucagon (green) and OX-8 (red) in cell suspensions cultured without r.IFN-gamma shows some non-specific binding may be present. X 625

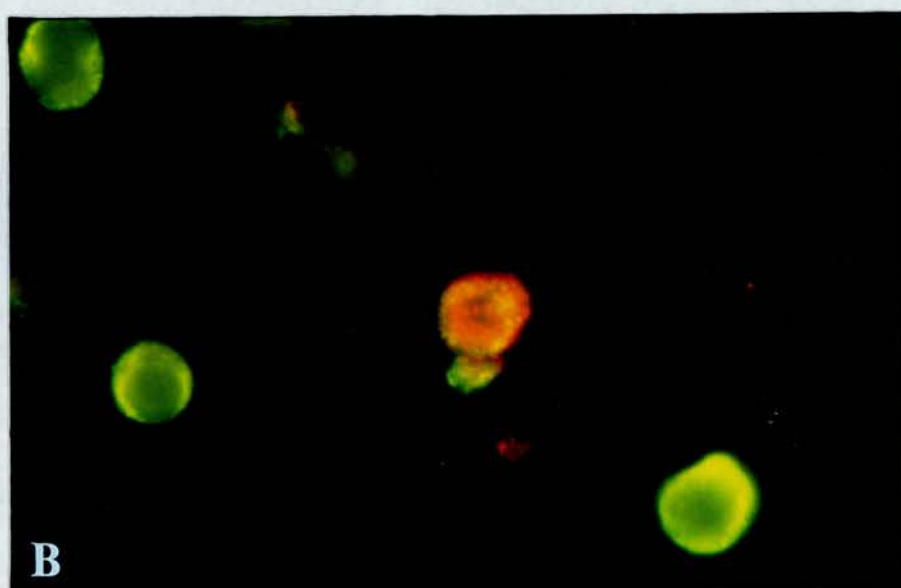
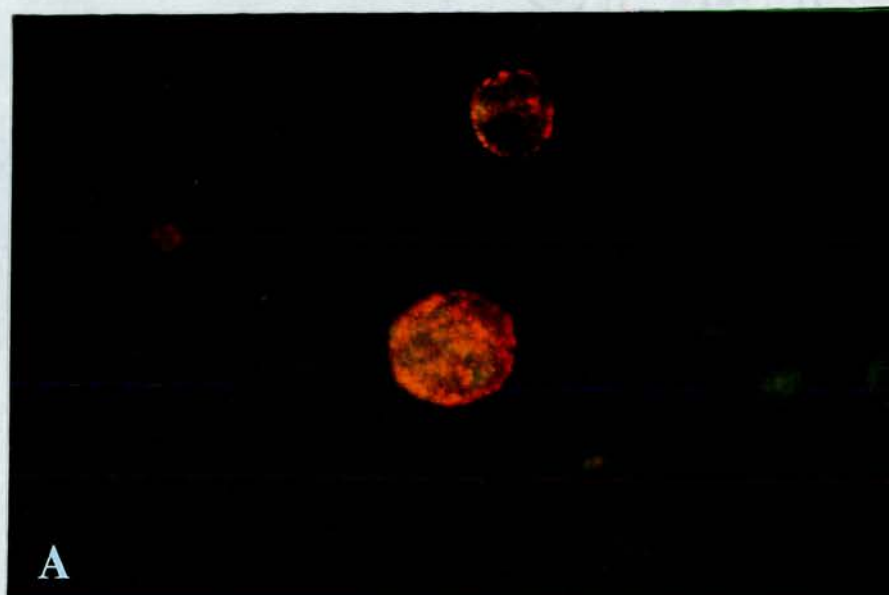


Fig 26 : MHC class I molecule expression on dispersed islet cell suspensions from 30 day old DR BB/E rats. X 625

- (A) control MHC class I antigen levels after 3 days culture.
- (B) OX-18 staining after 3 days culture with 10U/ml of r.TNF.
- (C) Class I MHC antigen expression following 3 days culture with r.TNF (10U/ml) plus r.IFN-gamma (100U/ml).

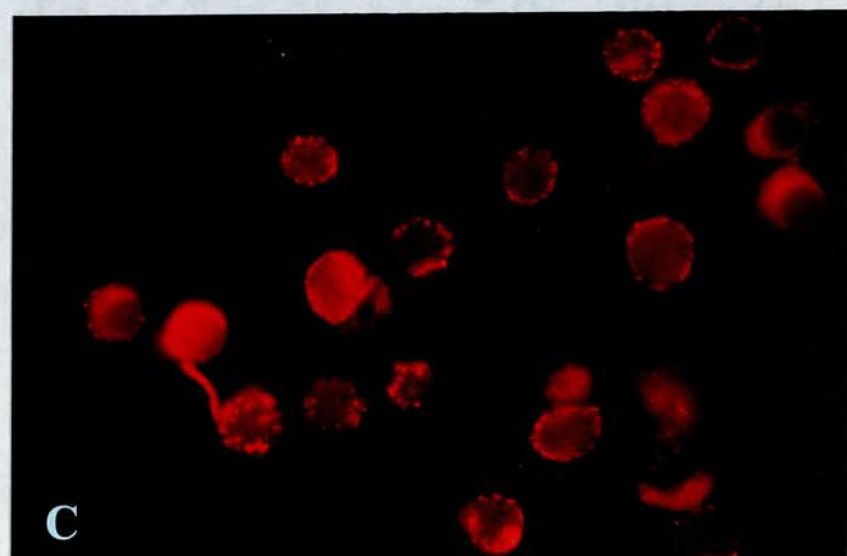
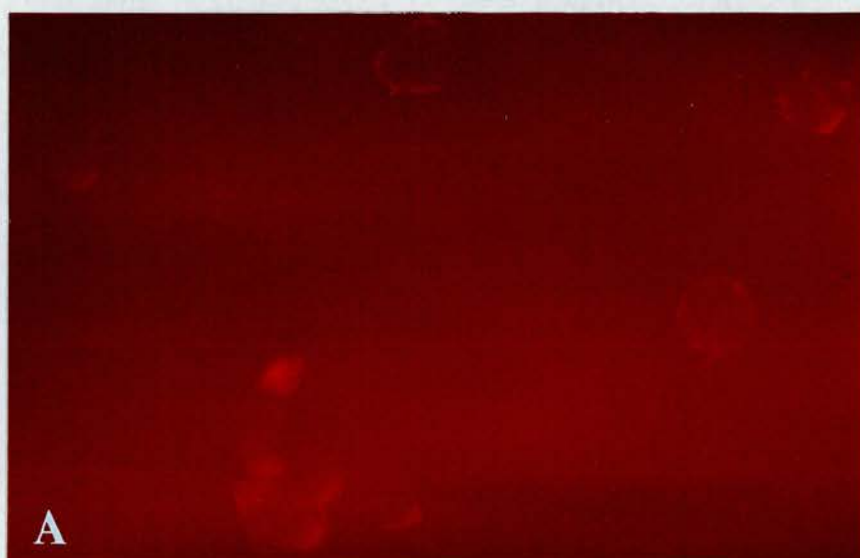
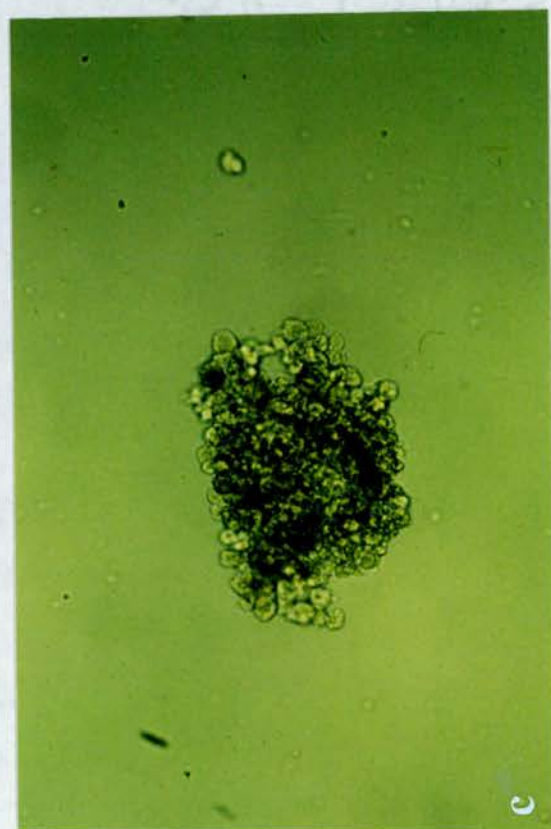
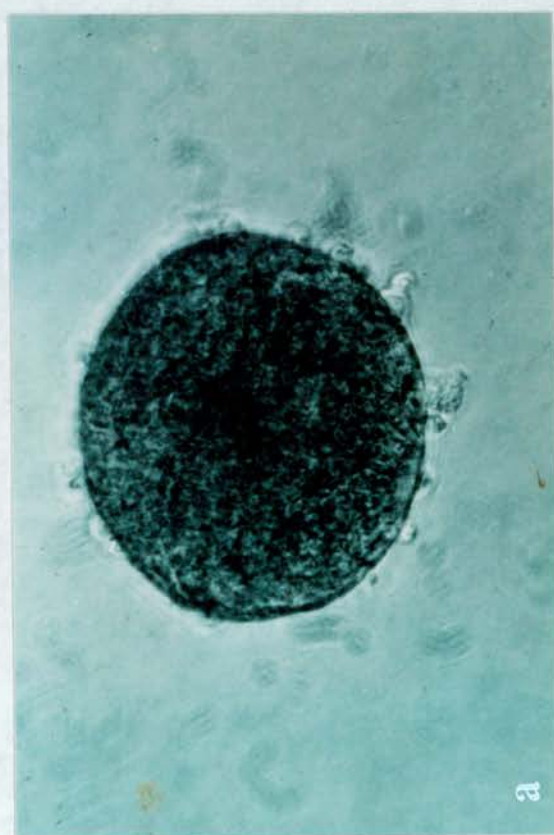
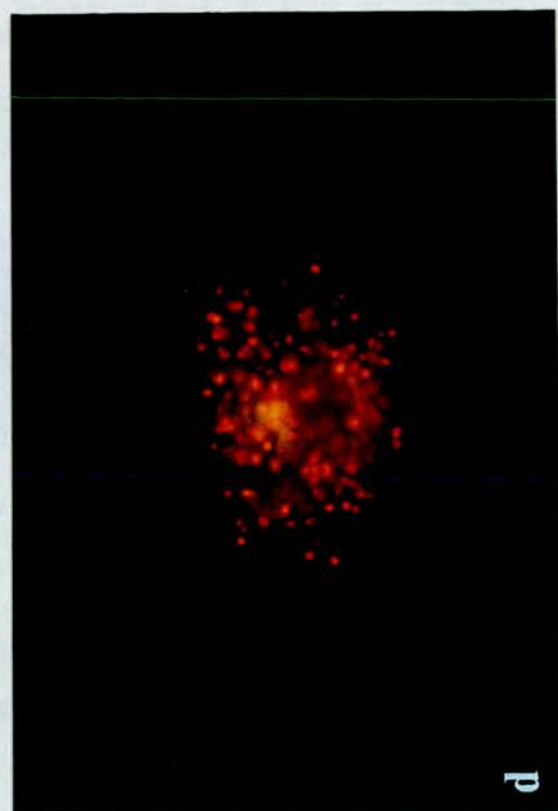
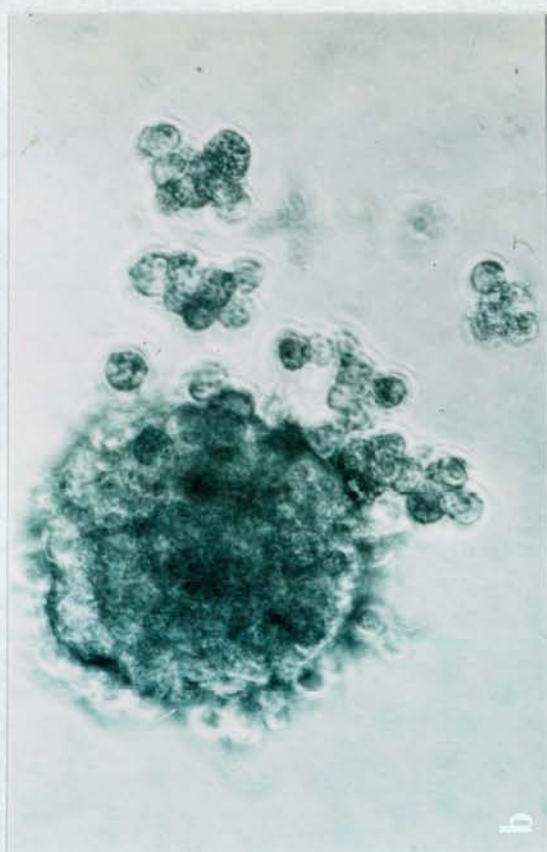


Fig 27 : "Whole" normal Wistar rat islets (aged 90 days) cultured for 5 days in the presence of r.IL-1 (20U/ml) or r.TNF (100U/ml).

- (a) control cultures. X 312
- (b) islets cultured with r.IL-1 show disruption of islet cell membranes. X 312.
- (c) islets cultured with r.TNF. X 125.
- (d) acridine orange/ethidium bromide staining of (c) indicates islet cell death.



DISCUSSION

Although IFN-gamma has been shown to induce MHC class II molecules on a wide variety of cell types (550, 551, 644-646), the studies herein described, in agreement with other studies (591, 592, 647, 649), indicate that islet beta cells are not readily stimulated by IFN-gamma to express class II MHC antigens.

It was found that islets isolated from DP BB/E rats aged 80-90 days, when cultured with 100U/ml of rat r.IFN-gamma for >4 days, expressed MHC class II molecules on approximately 50% of the insulin-containing beta cells. In contrast, induction of class II expression by r.IFN-gamma was never observed on (i) glucagon- or somatostatin-containing cells in islets from any animals; (ii) beta cells from either normal Wistar or DR BB/E rats, or (iii) beta cells from young (30 days of age) DP BB/E rats.

In the older DP BB/E rats, the presence or absence of ICSEA did not appear to be related to the sensitivity of the beta cells to r.IFN-gamma. However, since circulating ICSEA levels fluctuate in the prediabetic period, it is likely that some animals categorised as ICSEA negative, had been ICSEA positive at some time previously. Since expression of class II MHC antigens was not observed on any endocrine cells cultured in the absence of r.IFN-gamma, the expression of class II molecules specifically on beta cells isolated from DP BB/E rats, is unlikely to be due to adsorption of MHC class II proteins, shed from passenger macrophages.

Furthermore, islet cell suspensions stained with MoAbs against macrophages did not identify any significant macrophage contamination of the cultures. Indeed, this is not unexpected, since maintenance of isolated islets in free-floating culture for several days has been shown

to act as a purification step for removal of non-endocrine cells (663). By the same arguments, the observed induction of class II MHC molecules on DP BB/E beta cells is unlikely to represent phagocytic uptake of fragmented beta cells by macrophages which are themselves expressing class II MHC antigens (552, 553).

Since expression of class II molecules was only found on approximately 50% of the isolated beta cells, it is possible that the susceptibility of these beta cells was related to their differentiation state. Thus, the systemic induction of class II molecules on previously negative parenchymal and stromal cells, following intravenous infusions of r.IFN-gamma, appeared related to the differentiation stage of each cell population (664). Equally, the effect of IFN-gamma, on induction of class II antigens on various cell lines, appeared related to their differentiation state (645). This could be related to the methylation state of the DNA since treatment of certain non-responsive cells with 5-azacytidine, a DNA-demethylating agent, results in acquisition of susceptibility to IFN-gamma induced Ia expression (665). Accordingly, previous autoradiography studies in the DP BB/E rat have indicated an increase in islet cell replication in pancreatic sections obtained from animals of similar ages to those from which the islets susceptible to r.IFN-gamma induction of class II molecules were isolated (538).

Although most studies indicate that islet cells are refractory to the induction of MHC class II antigen expression by IFN-gamma alone, a few studies have reported class II induction on normal islets with IFN-gamma alone (648, 650, 651). In the studies by Wright et al (650, 651), longer culture periods and 10-20 fold higher concentrations of IFN-gamma were used when compared with our studies. Thus, culture of mouse

islets for >6 days (650) or human islets for 10 days (651), in high concentrations ($>10^3$ U/ml) of r.IFN-gamma, induced class II molecules on 15-20% of islet parenchymal cells, which included both beta and non-beta cells. Culture with a 5% lymphokine supernatant isolated from a T-cell line sensitized to *Listeria monocytogenes*, induced class II antigens on 10-40% of dispersed islet cells cultured for >9 days (650). However, the lymphokine supernatant could contain cytokines other than IFN-gamma, which may act to influence the islet class II expression. In the report by Markmann et al (648), mouse r.IFN-gamma (500U/ml) cultured for 6 days with islets isolated from Lewis rats, induced MHC class II proteins on the surface of whole islets. However, immunohistological staining for endocrine hormones was not performed and therefore the identity of the class II positive cells was not established.

The induction of MHC class II proteins on beta cells from DP BB/E rats, could suggest that the genotype of the diabetes-prone animals may confer a greater susceptibility to gamma-IFN induction of class II antigens, than the genotype of diabetes resistant animals. However, the failure to induce class II molecules on beta cells isolated from young DP BB/E rats indicated that the possession of a particular genotype was in itself not sufficient; and suggested the possibility that prior in vivo cytokine-mediated damage to the islets isolated from the older DP BB/E rats may have sensitized them to subsequent IFN-gamma induced class II expression. Since the mean age at onset of IDDM in the BB/E colony is about 96 days, and insulinitis appears at least 2 weeks before disease onset, it is likely that some of the islets isolated from the older DP BB/E rats would have already been damaged by infiltrating immunocytes.

The possibility that at least two signals were necessary to induce MHC class II antigens on islet cells was supported by other studies. In these studies on human (592, 652) and mouse (592) islets, whilst IFN-gamma alone was without effect, when combined with TNF or lymphotoxin (LT), induction of MHC class II molecules was observed on 30-50% of islet beta cells.

Tumour necrosis factor (TNF-alpha) is a cytokine primarily produced by activated macrophages (653), although T cells can also produce this form of TNF in addition to LT (666). Equally, amongst a variety of cell types (667); macrophages and T cells can synthesize and secrete interleukin-1, which has been reported to mediate islet cell damage in vitro (411, 412). Thus, since macrophages and T cells are prominent in the insulinitis of BB/E rats, it is likely that TNF and IL-1 are being produced in the islet microenvironment. Furthermore, synergy between IFN-gamma and these cytokines, have been well documented for a variety of effects (668), including; enhancement of cytotoxicity against either tumour cells (669) or pancreatic islets (413, 670); augmentation of antiviral activity (671) and amplification or induction of MHC antigens on endothelial (672) or thyroid (673) cells. Consequently, TNF and IL-1 seemed the most likely candidates to combine with IFN-gamma to induce MHC class II antigens on islet beta cells.

However, in our culture systems neither r.TNF or r.IL-1, either individually or in combination with r.IFN-gamma, were able to induce class II antigens on islet beta cells isolated from DR BB/E rats or normal Wistar rats aged 30 days.

These results are disparate from those of Pujol-Borrell et al (652) and Campbell et al (592) which describe the induction of MHC class II antigens on 30-50% of islet beta cells following culture with TNF or LT in combination with IFN-gamma. In the former study, human pancreatic monolayer cultures containing >80% non-endocrine cells, including ductal cells, macrophages and fibroblasts were used. Therefore, it is possible that the induced class II expression could have been potentiated via cytokines other than TNF released by other cell types within the monolayers.

In the latter study, isolated human and mouse islets were used, but induction of class II MHC proteins required using concentrations of 500-1000U/ml of both r.TNF and r.IFN for 5-6 days. In both studies, however, the induction of class II MHC molecules was not specific for the beta cells, being found also on the glucagon and somatostatin cells (652) and some unspecified non-beta cells (592). Accordingly, the results are at variance with the in vivo observations on pancreata from IDDM patients, where induction of MHC class II antigens is specific to the islet beta cells (261, 549).

Interestingly, in the above study by Campbell et al (592), the same combination of TNF and IFN-gamma when added to cultures of RINm5F cells, failed to induce MHC class II molecules on the cells. This could further suggest that the differentiation state of the beta cell might determine its susceptibility to cytokine induced expression of class II MHC proteins. This is supported by the findings of Varey et al (662); whereas freshly cultured RINm5F cells were readily induced by IFN-gamma to express class II MHC antigens, the induction was not so pronounced when long-term cultured cells were used.

The failure to induce MHC class II antigens on normal Wistar or DR BB/E rats with combinations of r.TNF, r.IL-1 and r.IFN-gamma suggests that this particular triad of cytokines do not, at least in vitro, act to induce class II antigens on normal rat islets. However, it may be that where more than one signal is required, the different signals have to be delivered in an appropriate sequence. Thus, the susceptibility of thyroid epithelial cells to the cytotoxic action of TNF is enhanced by incubating the cells with IFN-gamma before exposure to TNF (674). This synergy between IFN-gamma and TNF may ensue following induction of TNF receptors on cells by preincubating the cells with IFN-gamma (675). In our experiments, when combinations of cytokines were used, they were added simultaneously. Therefore, it cannot be excluded that different results might have been obtained if, for example, the islets had been preincubated with IFN-gamma and then incubated with TNF or IL-1.

Synergistic effects between TNF and IFN-gamma were however observed as regards their effects on islet class I MHC expression. In agreement with Campbell et al (592), incubation of islets from both normal Wistar and DR BB/E rats, with r.TNF alone, enhanced the expression of MHC class I proteins on all endocrine cell types. Addition of r.IFN-gamma further augmented the levels of islet class I expression. Similar enhancement of MHC class I proteins, by TNF, have been reported for human endothelial cells (672), rat thyroid cells (673) and rat astrocytes (676).

Cytotoxic and cytostatic effects of TNF (674, 677) and IL-1 (678, 679) against a variety of normal and transformed cells have been documented. Both TNF (670) and IL-1 (411) have been shown to exert cytotoxic effects towards cultured islet cells. Furthermore,

reports on the synergism between these cytokines as well as with IFN-gamma; causing enhanced islet cell cytolysis, have been described (412, 413, 670).

As an adjunct to our experiments, it was noticed that islets cultured with r.TNF (>50U/ml) or r.IL-1 (>10U/ml) either singly or in combination with r. IFN-gamma (100U/ml), showed evidence of morphological damage after 4 days in culture. After 3 days of culture, with 2000U/ml of murine r.TNF, inhibition of glucose-stimulated insulin release from isolated mouse islets was reported by Campbell et al (670). This effect was augmented by the addition of murine r. IFN-gamma, such that after 6 days of culture with both cytokines, complete morphological disintegration of the islets was observed (670).

Using a ^{51}Cr -release assay and rat pancreatic islets in monolayer culture, Pukel et al (413) did not observe any cytotoxic effects on the monolayers with human r.TNF (100U/ml) or human r.IL-1 (10U/ml) alone. However, combinations of these cytokines with each other or rat r. IFN-gamma proved cytolytic to the monolayer cultures, with maximal effect obtained with the combination of all three cytokines. Where human r.TNF was used on rat islets (412, 413), the failure to detect TNF mediated islet cytotoxicity, could suggest that to some degree this effect is species specific (680). Thus, in the studies by Pukel et al (413), both rat and mouse, but not human r. IFN-gamma exerted a small dose dependent lysis of the rat islet monolayers. In our studies however, both human and murine r.TNF were used; and both were capable of disrupting the morphology of the free-floating rat islets.

Over the last few years several reports, mainly from Jorn Nerup and his group at the Steno Memorial Hospital in Denmark, have described the cytotoxic effects of IL-1 on isolated islets (411, 412, 681-684) and these have recently been reviewed (685).

Initially, culture of rat or human islets for 7 days with a crude supernatant obtained by stimulating peripheral blood mononuclear cells from healthy human donors with antigen or PHA, was found to affect glucose-stimulated insulin secretion, and reduce the islet insulin and glucagon content (681). Subsequently, the identity of the cytotoxic activity was determined when adsorption with a purified antibody to IL-1 eliminated the cytotoxic activity from the crude supernatant (682). Thereafter, the addition of highly purified human IL-1 to cultures of isolated rat islets for 6 days led to inhibition of glucose-induced insulin release, reduction of islet contents of insulin and glucagon, and disintegration of the islets (411). Factors which reduced the IL-1 islet cytotoxicity included, increasing the glucose concentration in the culture medium, and the addition of normal human or rat serum, thereby indicating the presence of IL-1 inhibitors or antagonists within normal serum (683).

These inhibitory effects of IL-1 on islet insulin secretion have been corroborated by other groups (686-688). However, in addition to its inhibitory effects, IL-1 at lower concentrations (20-26 ug/l) can also potentiate glucose-stimulated insulin release (689). Therefore, depending on the parameters of IL-1 concentration and duration of exposure, IL-1 can be stimulatory or inhibitory for glucose-induced insulin release from isolated islets (684, 686, 687, 690). Thus, Comens et al (687) found that IL-1 (5pM) incubated for 15h, stimulated glucose-induced insulin secretion,

whereas a similar incubation with a 10-20 fold greater IL-1 concentration was inhibitory. Equally, the larger doses given for shorter time periods (90 mins), also proved stimulatory. The inhibitory effects were not due to beta cell death since islets were morphologically intact, displayed normal islet plasma membrane permeability, and the effects were reversible following removal of IL-1 (687). Thus IL-1 in short term cultures can influence beta cell function without inducing cell death.

It has been proposed that IL-1 may be involved in the pathogenesis of IDDM by mediating selective destruction of the pancreatic beta cells (685). However, it is contentious whether or not IL-1 induced islet cytotoxicity is specific for the beta cells. In their initial studies (411, 681, 682, 684), Mandrup-Poulsen and colleagues found that incubation of islets with IL-1 in addition to affecting insulin synthesis and secretion, also reduced the glucagon content of the islets. However, in a more recent time course study, ultrastructural analysis revealed that whereas beta cells were already subjected to pathological changes after 30 mins. exposure to 25U/ml of IL-1, no changes were observed in glucagon cell morphology during 24h exposure (691). Thus it is suggested that a short half-life in vivo, together with a high local IL-1 concentration, may allow for the selective destruction of islet beta cells by IL-1 (685).

In conclusion, our in vitro studies on the ability of cytokines to modulate expression of MHC antigens on isolated islets, are compatible with our in vivo findings, whereby expression of MHC class II molecules on pancreatic beta cells represents a secondary phenomenon, probably influenced by release of cytokines from infiltrating macrophages and lymphocytes. In

general, islet beta cells appear refractory to the induction of class II MHC proteins by cytokines.

The preferential induction of class II antigens on beta cells isolated from DP BB/E rats, suggested that genotype could influence islet cell susceptibility to the effects of various cytokines. Thus, murine JHM Coronavirus, induced Ia expression in rat strains susceptible to JHM virus-induced demyelinating disease, but not in rat strains resistant to the disease (692). However, Mandrup-Poulsen et al (683) reported that genetic background did not influence the susceptibility of isolated rat islets to the cytotoxic effects of IL-1, with no differences observed amongst four inbred rat strains. This study did not however include any diabetes-prone rats or rat strains susceptible to other autoimmune diseases.

Subsequently, Anderson et al (693) isolated islets from DP and DR BB rats, as well as from three inbred non-diabetic rat strains and cultured them with human r.IL-1 for 7 days. Strain related differences in the sensitivities of the islets to the cytotoxic effects of IL-1 were detected, but these differences were unrelated to the propensity to develop IDDM. However, the study used newborn rat islets, and therefore it is possible that detection of strain-related differences to the effects of IL-1 may be missed. Furthermore, cyclosporine (CSA) was given to the pregnant diabetes-prone rats in order to prevent them developing diabetes during pregnancy. Since CSA can also inhibit insulin synthesis and secretion, i.e. similar to IL-1 (694), this may alter the IL-1 sensitivities of the islets isolated from the offspring of these animals.

Alternatively, the differentiation state or the activation state of the islets at their time of isolation, may be important in determining their sensitivity to the effects of cytokines. Thus, Palmer et al (695) have recently shown that the resting beta cell is more resistant to IL-1 mediated cytotoxicity than the stimulated beta cell.

Although many studies have investigated the effects of cytokines on islet MHC antigen expression or cytokine mediated islet destruction, their results have not always been in agreement. These disparate results indicate that in attempting to mimic the in vivo situation, in vitro studies have limitations which necessitate that caution must be exercised in extrapolating their results to the in vivo situation. Accordingly, variations in the source, species and concentration of cytokines employed, combined with differences in the culture conditions and duration of cytokine exposure, will produce varying results between different centres. Furthermore, in vivo, the levels of cytokine production are likely to be under genetic control (654), and the network of their interactions with each other, producing synergistic or antagonistic effects on target cells are also likely to be finely controlled, such that it is unlikely that in vitro studies can accurately mimic the in vivo situation.

**CHAPTER 8 - EFFECT OF CYCLOSPORINE ON THE DEVELOPMENT
OF IDDM IN THE BB/E RAT.**

INTRODUCTION

Cyclosporine (CSA) is a small hydrophobic cyclic undecapeptide derived from the fungus, *Tolypocladium inflatum* Gams, and is widely used as a potent immunosuppressive agent (696, 697). Its immunosuppressive properties were first described by Borel et al (698, 699). Cyclosporine inhibited humoral immunity as shown by a reduction of plaque forming cells, and suppressed cell-mediated immune responses by preventing skin graft rejection and inhibiting the development of GVHD and EAE in rodents (698). In addition, CSA blocked delayed type hypersensitivity (DTH) responses to tuberculin and oxazolone (699). Its failure to prohibit antibody synthesis to LPS in nude mice, suggested a selective effect on T lymphocytes (699). However, subsequent studies have demonstrated CSA mediated suppression of B lymphocyte activation by anti-Ig antibodies (700).

Cyclosporine can affect both T_H (699, 701) and T cyt (702) cells, but T supp cells are reportedly resistant to its actions (702, 703).

However, it is suggested that the effect of CSA on the generation of T cyt cells is a consequence of its primary effect on T_H cells (701). This is assumed to result following blockage of lymphokine production by CSA (704). Impairment of IL-2 production in mixed lymphocyte cultures (701, 705) and following mitogen stimulation (706), has been demonstrated. This blockade of IL-2 production by CSA, has been shown to act at the mRNA level (707, 708), where CSA acts after antigen binding and before transcription of lymphokine encoding mRNA (709). Thus, in vivo, CSA does not appear to inhibit the initial process of T cell priming by antigen (710, 711), but since CSA affects lymphokine synthesis, the priming event does not result in observable in vivo reactions, provided that inhibitory levels of CSA are

maintained. However, CSA cannot block the translation of lymphokine mRNA once it has been formed in the cell (712). Therefore, once T cells have been activated to produce message for lymphokine synthesis, they become insensitive to CSA. Thus, clonal expansion of activated T cells by preformed IL-2 is unaffected by CSA (713).

In addition to its effects on IL-2 production, CSA has also been shown to inhibit the production of other lymphokines and cytokines. The generation of lymphokines affecting macrophage activities, e.g. migration inhibition factor, lymphocyte-derived chemotactic factor and procoagulant-inducing factor, are blocked by CSA (714). Cyclosporine mediated inhibition of IL-1 release from monocytes and macrophages (701, 715) and from a human macrophage cell line (706), have been demonstrated. However, Wagner (716) failed to observe suppression of IL-1 release from macrophages in response to LPS and suggested that CSA may only inhibit T cell-dependent IL-1. Equally, CSA can also affect the production of TNF (717) and IFN-gamma (718).

Since CSA does not display significant toxicity towards haemopoietic tissues (699), but inhibits lymphokine-dependent immune responses, whilst sparing non-specific defense functions such as the phagocytic activity of macrophages (719), it has widely become the agent of choice in the clinical management of graft rejection (720). More recently, CSA has been used in the treatment of certain autoimmune diseases (721, 722) including uveitis (723), Graves ophthalmopathy (724), some skin disorders (725) and IDDM (726).

Laupacis et al (427) was the first to demonstrate that CSA administered to young (30 day old) DP BB rats could completely prevent the development of IDDM. This success prompted Stiller et al (727) to perform an

uncontrolled trial on the effects of CSA on recently diagnosed IDDM patients. Whereas 16 of 30 patients treated within 6 weeks of diagnosis became insulin independent, only 2 of 11 (18%) who entered the study 8-44 weeks after diagnosis went into remission. Although this open pilot study did not control for the possibility of spontaneous remissions, it provided sufficiently beneficial results to encourage further studies. Accordingly, two placebo-controlled double-blind trials were subsequently initiated (431, 728).

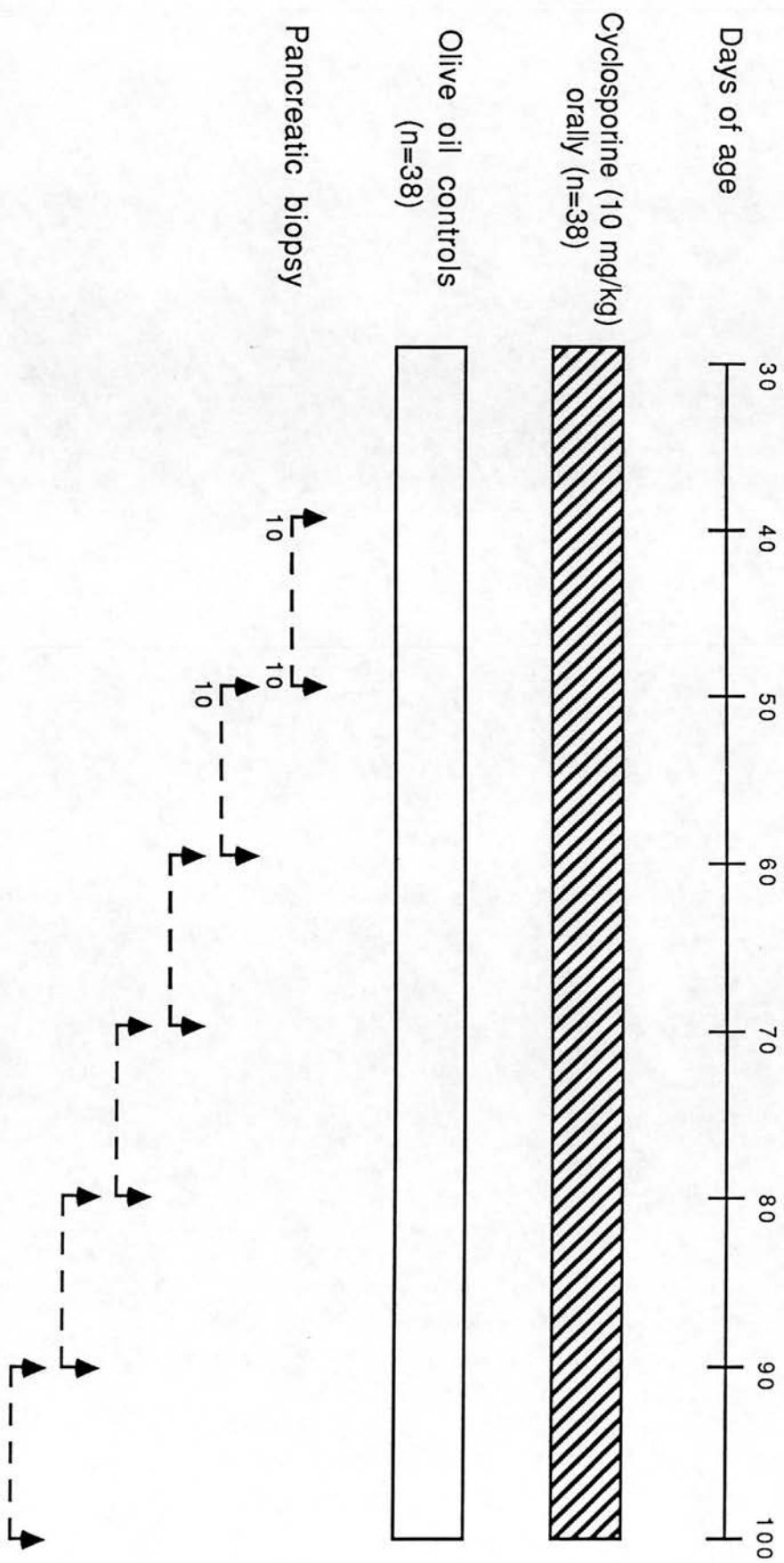
In the French multicenter trial (431), CSA was initially given to 122 patients at a dose of 7.5mg/kg/day for 3 months, which in the absence of remission was increased to 10mg/kg/day before systematically reducing to 5mg/kg/day after 6 months. The rates of complete remission at 6 months and 12 months of treatment were 37.5% and 32.3% respectively, compared with 18.6% and 0% for the placebo group. The Canadian-European trial (728) administered CSA (10mg/kg/day) for 12 months to 188 patients and obtained a 38.7% and 24.2% remission rate after 6 and 12 months respectively as compared to 19.1% and 9.8% with placebo. If CSA was administered within 6 weeks of diagnosis, the remission rates increased to 55.3% and 31.6% after 6 and 12 months of treatment. Both studies however indicated that withdrawal of CSA treatment was associated with the return of progressively increasing insulin requirements. Thus, maintenance of remission necessitated continual administration of CSA.

Following the initial report by Laupacis et al (427), further studies of CSA treatment in BB rats have produced a spectrum of findings, including: (i) complete prevention of diabetes (428); (ii) reduced frequency and delayed onset of disease (426, 429, 729, 730); and (iii) no effect on the incidence of hyperglycaemia, but

producing a milder diabetic syndrome (430). Thus, although there are some indications that CSA may be of benefit in the treatment of Type 1 diabetes in the BB rat, the mechanism of its action in inhibiting the autoimmune destruction of the beta cells has not been defined.

Therefore, we have used serial pancreatic biopsy to monitor the effect of CSA on the development and progression of diabetes in individual DP BB/E rats followed for the duration of their natural life-span. In relation to the discrepancies between previous studies, it was important to address the questions - Does CSA treatment: (i) prevent or merely delay the onset of diabetes? (ii) improve the prognosis after disease onset? and (iii) produce significant side effects?

Figure 28 : Cyclosporine Biopsy Study - Protocol



MATERIALS AND METHODS

ANIMALS

DP BB/E rats from 11 litters were divided into two groups (N=38 per group) and subsequently treated orally from 30-100 days of age with either CSA (10mg/kg body weight/day) or olive oil vehicle. Cyclosporine was kindly donated by Dr J F Borel (Sandoz Ltd, Basle, Switzerland). Body weight and urinary glucose were measured daily in all animals during and after treatment to detect the onset of IDDM. Animals developing diabetes were allocated alternatively to treatment with a single daily subcutaneous injection of insulin (Ultratard bovine insulin, Novo, Copenhagen, Denmark) only, or insulin and oral CSA (10mg/kg/day) for 50 days. The dose of insulin was adjusted individually on the basis of daily measurement of body weight and glycosuria in both groups of diabetic animals. Animals not developing IDDM were followed to the end of their natural life span.

PANCREATIC BIOPSY

Pancreatic biopsy was performed, as described in Chapter 6, on groups of CSA treated (N=5 per group) and oil treated (N=5 per group) rats between 40 and 100 days of age. Each rat was biopsied twice with a 10 day interval (Fig. 28). Animals developing IDDM were biopsied for a third time at the onset of disease and a fourth biopsy was taken at sacrifice after 50 days of treatment with either insulin alone or insulin and CSA. Animals not developing IDDM were also biopsied for a third time to provide age matched control samples for the biopsies taken from diabetic animals 50 days after the onset of diabetes.

Tissue obtained at biopsy was immediately snap frozen for subsequent immunohistological examination. Blood samples were also obtained from the tail vein at the time of each biopsy and assayed for plasma glucose (glucose analyser - Beckmann Instruments, Fullerton, CA) and circulating white blood cells (WBC). In addition, ICSC and IAA were measured as previously described.

IMMUNOHISTOLOGY

Cryostat sections from the pancreatic biopsies were screened with antibodies directed against rat MHC antigens, lymphocyte and macrophage subsets (Table I), and islet endocrine hormones and detected by indirect immunofluorescence procedures as described in Chapter 4.

OTHER ASSAYS

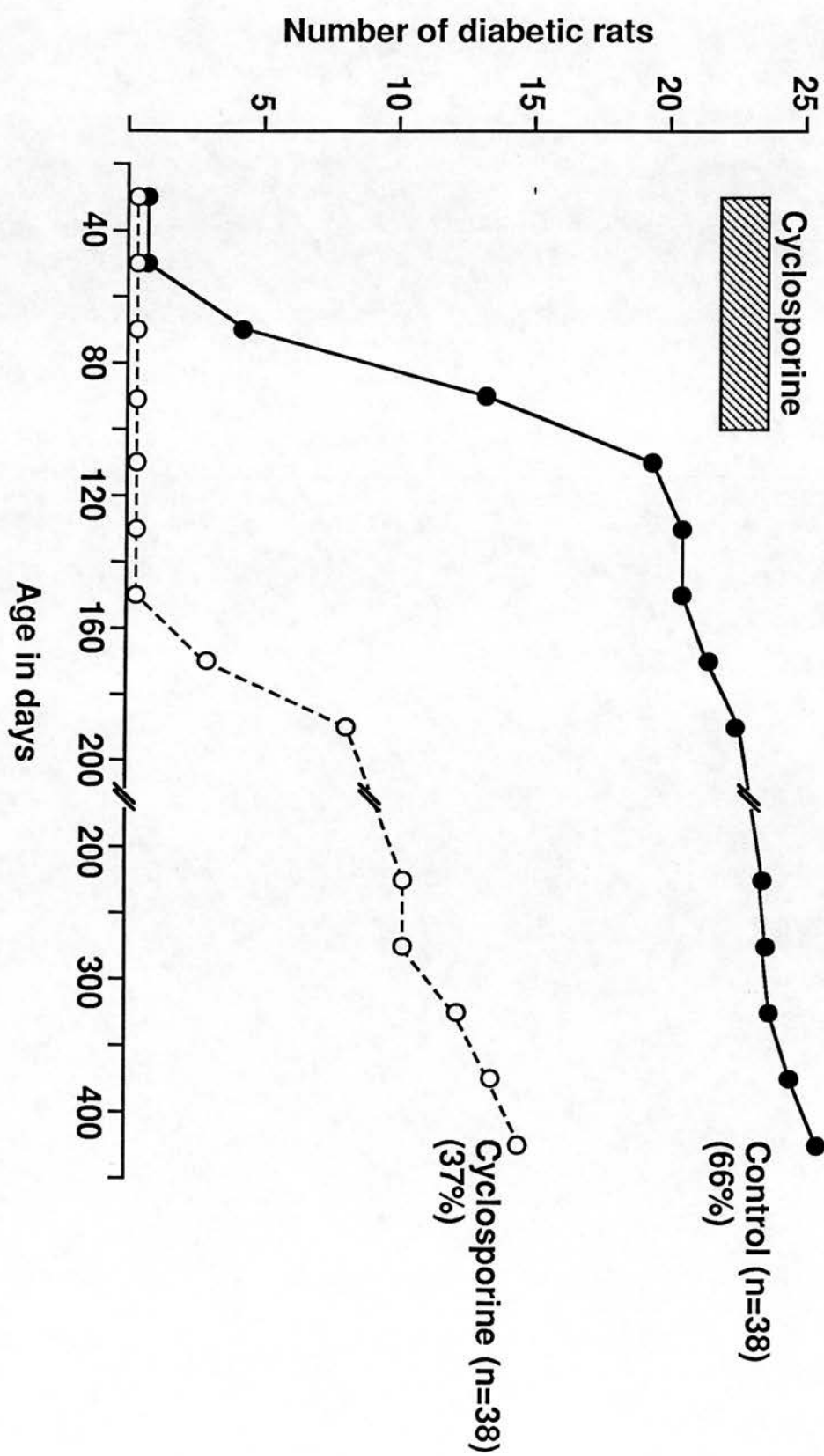
Tail vein blood samples (0.1ml) were extracted with 2ml of ice cold 2.5% (w/v) trichloroacetic acid. Samples were centrifuged immediately at 4°C (1500g for 20 mins.) and the supernatant stored at -20°C. Blood glucose, lactate, glycerol, alanine and 3-hydroxybutyrate were assayed in the supernatants using modified enzymatic fluorimetric methods (731) by Dr J Burrin (Hammersmith Hospital, London).

Enzymatic determination of plasma cholesterol and triglycerides were performed by calorimetric detection methods on a technicon autoanalyzer using test-kits obtained from Boehringer Mannheim (Lewes, East Sussex, England).

Plasma creatinine was measured by a Creatinine Analyzer (Beckmann Instruments Incorporated, Fullerton, California).

White blood cell counts were measured on blood samples obtained from the tail vein. Whole blood (0.1ml) was mixed with 0.2ml of distilled water; whirlmixed for 15 secs. to lyse the red blood cells, after which 0.2ml of HBSS was added before centrifugation at 4°C (200g for 10 mins.). The cell pellet was resuspended in HBSS (0.1ml) and WBC numbers estimated on a haemocytometer following acridine orange/ethidium bromide staining of the cell suspensions.

Figure 29: Effect of cyclosporine on development of IDDM



RESULTS

The development of diabetes in the oil treated control and CSA treated rats, is shown in Fig. 29. During the treatment period (30-100 days of age), 13 of the oil treated control rats developed IDDM compared with none of the CSA treated animals. By 150 days of age, 20 control rats were diabetic and there were still no diabetic animals in the CSA treated group. Between 150 and 200 days of age, however, 8 animals that had previously received CSA, developed diabetes along with one more control animal. By 450 days of age, a further 4 control animals and 6 CSA treated rats became diabetic. No further cases of diabetes occurred in either group thereafter, giving a final incidence of diabetes of 66% (25/38) and 37% (14/38) with the mean ages at onset; 114 ± 16 days (\pm SEM) and 223 ± 21 days in control and CSA treated groups respectively.

Thus, treatment with CSA in the prediabetic period both postponed the onset and reduced the overall incidence of diabetes in DP BB/E rats.

During the treatment period, whereas IAA were detected in 6 of 38 (16%) animals receiving CSA, ICSA were not found in any of the CSA treated rats. However, the subsequent development of IDDM following withdrawal of CSA therapy, was associated with the corresponding appearance of ICSA (Table XIII). Islet cell surface antibodies were identified in 31 of 39 diabetic and 5 of 37 non-diabetic study animals. The incidence of ICSA was similar in oil treated (20/25) and CSA treated (11/14) rats at the onset of diabetes. Circulating IAA were found with similar frequency ($15 \pm 2.1\%$; mean \pm SEM) in diabetic and non-diabetic animals irrespective of the treatment regime.

The effect of CSA on circulating intermediary metabolite levels in non-diabetic DP BB/E rats, towards the end of the treatment period, is compared in Table XIV.

Although the plasma glucose concentration was significantly raised in CSA treated animals, it subsequently returned to control levels following discontinuation of CSA. Equally, although the concentrations of plasma triglycerides were elevated in CSA treated rats when compared with untreated DR BB/E rats, they were similarly raised in the oil treated controls (Table XV). Table XV also shows there was no difference in plasma creatinine concentrations between non-diabetic CSA treated, and oil treated DP BB/E rats, and age matched non-diabetic DR BB/E rats. In addition, administration of CSA to DP BB/E rats, did not significantly reduce their numbers of circulating white blood cells, when compared with their oil treated controls (Table XVI).

At onset of diabetes, the effects of administering insulin and insulin combined with CSA, on the subsequent progression of diabetes was assessed by monitoring daily insulin requirements and body weight measurements (Fig. 30). Administration of CSA, in addition to insulin, failed to induce remission of diabetes in any animal. Body weight gained during the 50 days post onset of hyperglycaemia was similar in both treatment groups: increasing from $295 \pm 31\text{g}$ (mean \pm SEM), to $327 \pm 30\text{g}$ in rats given insulin, whilst rats maintained on insulin with CSA increased from $279 \pm 28\text{g}$ to $326 \pm 28\text{g}$. The daily insulin dose required to maintain aglycosuria during the 50 day treatment period was similar in both groups (2.0 ± 0.13 and 2.0 ± 0.14 U/day; insulin and insulin/CSA respectively).

TABLE XIII: INCIDENCE OF CIRCULATING ICSA AND IAA IN RELATION TO
THE DEVELOPMENT OF IDDM

	<u>ICSA positive</u> <u>% (N)</u>		<u>IAA positive</u> <u>% (N)</u>	
	<u>Diabetic</u>	<u>Non-diabetic</u>	<u>Diabetic</u>	<u>Non-diabetic</u>
Olive Oil Treated	80 (20/25)	31 (4/13)	12 (3/25)	15 (2/13)
CSA Treated	79 (11/14)	4 (1/24)	21 (3/14)	12 (3/24)

TABLE XIV: EFFECT OF CSA TREATMENT ON PLASMA INTERMEDIARY METABOLITES
IN NON DIABETIC DP BB/E RATS

DP BB/E rats (70-80 days of age)	Intermediary Metabolites (mmol/l \pm SEM)				
	<u>Glucose</u>	<u>Lactate</u>	<u>3-OH Butyrate</u>	<u>Alanine</u>	<u>Glycerol</u>
Oil Treated (N=18)	8.7 \pm 0.3	1.35 \pm 0.06	0.36 \pm 0.05	0.75 \pm 0.04	0.23 \pm 0.02
CSA Treated (N=19)	9.6 \pm 0.3*	1.45 \pm 0.09	0.28 \pm 0.03	0.85 \pm 0.06	0.24 \pm 0.02

* p<0.025 Cyclosporine treated vs oil treated.

TABLE XV: EFFECT OF CSA TREATMENT ON PLASMA CONCENTRATIONS OF
CHOLESTEROL, TRIGLYCERIDES AND CREATININE IN
NON-DIABETIC BB/E RATS

	<u>Plasma Concentrations</u>		
	Cholesterol	Triglycerides	Creatinine
	(mmol/l \pm SEM)		(μ mol/l \pm SEM)
DP BB/E rats (100 days of age):			
Oil treated	3.25 \pm 0.08 (N=10)	3.67 \pm 0.80* (N=10)	46.9 \pm 1.6 (N=16)
CSA treated	2.92 \pm 0.07 (N=10)	3.70 \pm 0.41** (N=10)	44.1 \pm 0.9 (N=14)
DP BB/E rats (150 days of age) (N=6)	3.10 \pm 0.13	1.20 \pm 0.09	-
DR BB/E rats (100-120 days of age)	3.16 \pm 0.08 (N=10)	1.51 \pm 0.17 (N=10)	47.9 \pm 1.4 (N=13)

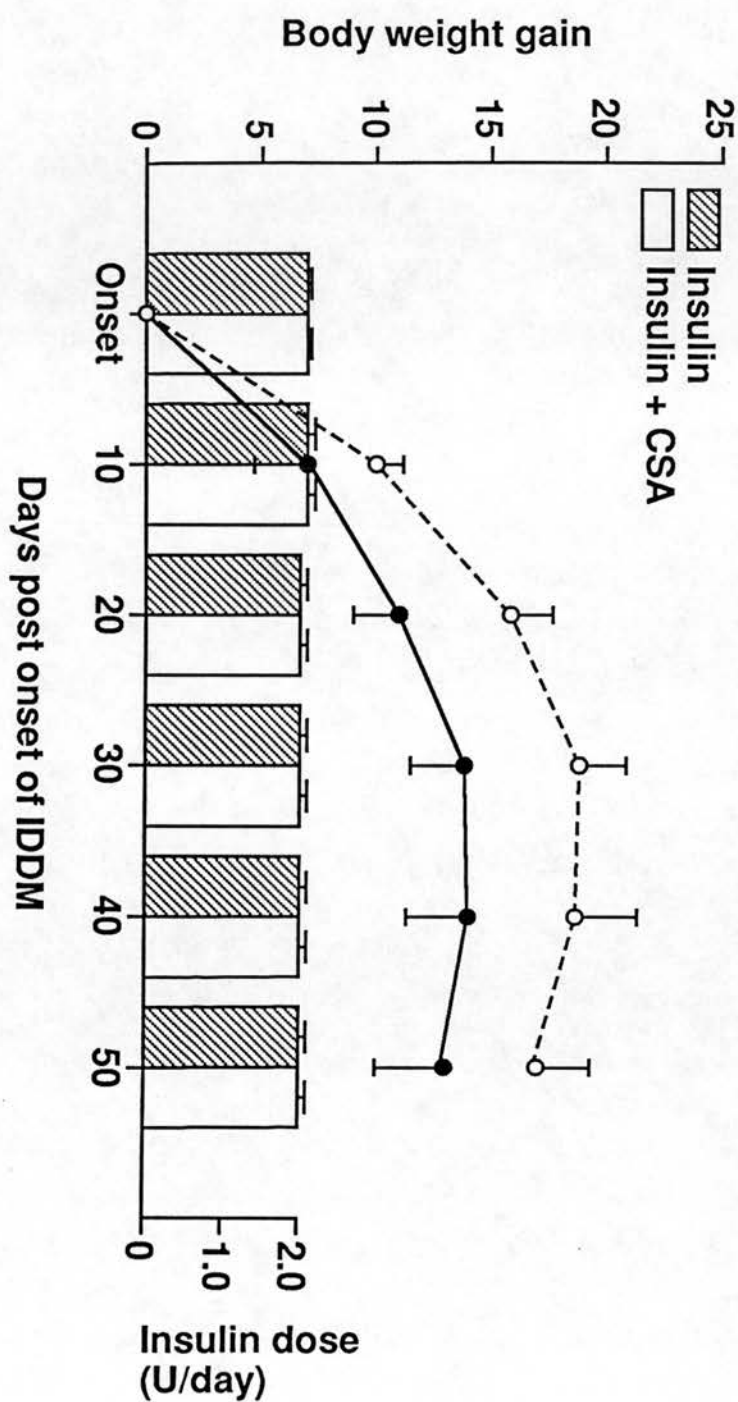
* p<0.01; ** p<0.0005 compared with DR BB/E rat group.

TABLE XVI: EFFECTS OF CSA TREATMENT ON LEVELS OF CIRCULATING
WHITE BLOOD CELLS

Rat Group	Treatment	WBCx10 ⁶ cells/ml (mean \pm SEM)
Non-diabetic DP BB/E rats (30 days)	Oil treated (N=38)	2.1 \pm 0.1*
	CSA treated (N=38)	2.2 \pm 0.1*
Non-diabetic DP BB/E rats (80-100 days)	Oil treated (N=25)	2.5 \pm 0.1*
	CSA treated (N=25)	2.3 \pm 0.1*
Diabetic DP BB/E rats at onset of disease	Oil treated (N=19)	2.7 \pm 0.3
	CSA treated (N=11)	2.6 \pm 0.5
Diabetic DP BB/E rats 50 days post onset	Insulin treated (N=13)	4.4 \pm 0.5*
	Insulin + CSA treated (N=13)	3.3 \pm 0.3
Non-diabetic DR BB/E rats (150-200 days) (N=20)	Untreated	3.0 \pm 0.1

* p<0.0005 compared to DR BB/E rat group.

Figure 30: Effect of cyclosporine after onset of diabetes



Immunofluorescence studies using MoAbs directed against rat MHC antigens and leucocyte subsets, on cryostat sections of pancreatic biopsies obtained from oil treated controls who developed IDDM either during or after the treatment period (30-100 days of age), produced similar results to those previously discussed in Chapter 6. Thus, the first change observed, occurred in some pancreatic lobules about 30 days before the onset of diabetes and consisted of increased expression of MHC class I molecules within islets and on the vascular endothelium. Subsequently, between 2-3 weeks before the onset of IDDM, expression of MHC class II molecules on vascular endothelium accompanied accumulation of ED1⁺ cells at periductal and perivascular sites. These cells then infiltrated the adjacent islets thereby initiating insulinitis. About 10 days before disease onset, increasing numbers of other immune effector cells were found infiltrating the islets. At this point, hyperexpression of MHC class I molecules within infiltrated islets was markedly enhanced, extending beyond the islet periphery into the surrounding exocrine tissue. At disease onset, although several of the islets presented with intense insulinitis and some residual insulin-containing cells, most of the islets presented without surviving beta cells and intra-islet infiltrates.

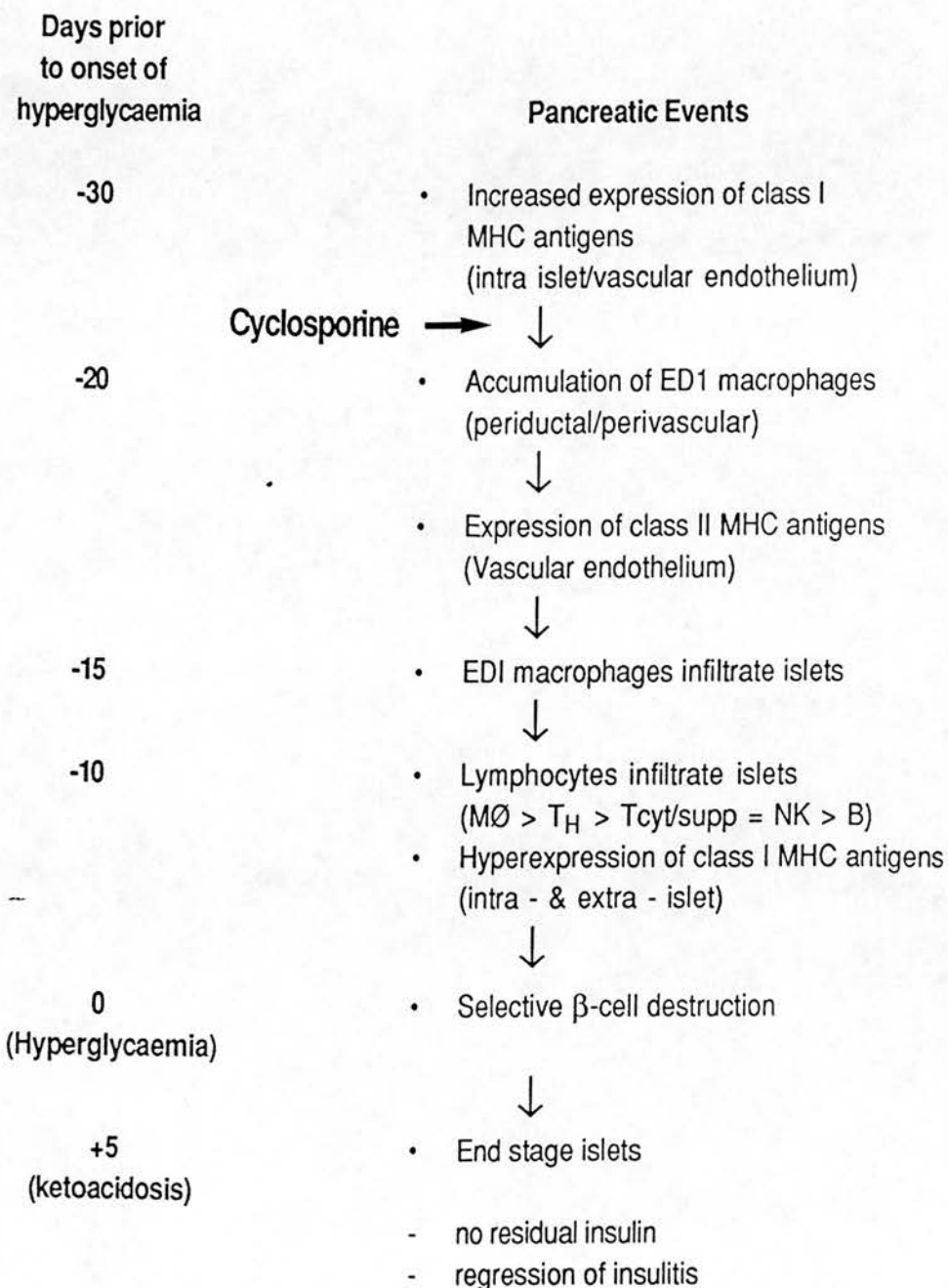
Biopsies taken from CSA treated rats during the treatment period showed a complete absence of insulinitis in all of the animals. At most, 10-15 ED1⁺ cells were occasionally observed around some of the larger blood vessels adjacent to islets. However, in biopsies taken from CSA treated animals during the treatment period, 11 animals showed enhanced intra-islet MHC class I expression of which 7 subsequently developed diabetes (mean age at onset = 176 ± 5 days (\pm SEM); and mean duration of time lapsed between observed increased islet

class I expression and disease onset = 100 ± 11 days). Thus, treatment with CSA in the prediabetic period had no effect on the expression of class I MHC antigens, but markedly inhibited accumulation of ED1⁺ cells at extra-islet sites, the subsequent recruitment of immune effector cells and islet infiltration (Fig. 31).

In biopsies taken from 6 animals that had previously been treated with CSA but had remained non-diabetic by 158 days of age, 2 animals presented with perivascular infiltrates and mild insulitis. Both these animals subsequently developed IDDM 10 days and 25 days later. The biopsies from the 4 remaining rats showed no evidence of pancreatic infiltration, insulitis or altered MHC class I expression. Three of these animals remained non-diabetic throughout their lives, whilst one became diabetic, but not until 400 days of age.

At onset of diabetes, biopsies removed from animals previously treated with CSA did not appear different from those similarly removed at onset from oil treated controls. Thus, there were no differences in the severity of insulitis or numbers of islets with residual insulin-containing cells. Following the diagnosis of diabetes, animals were either maintained on insulin or combined insulin and CSA treatment for 50 days. In the main, the biopsies taken at the end of this 50 day post disease onset period, showed no differences between the two groups. Thus, virtually all of the islets were end stage in appearance, with no observable infiltrates or enhanced MHC class I expression. However, in 3 of 13 biopsies removed from rats treated with insulin and CSA, there were several islets which still retained an infiltrate, some insulin containing cells and hyperexpression of class I MHC molecules.

FIGURE 31; EFFECT OF CYCLOSPORINE ON THE SEQUENCE OF PANCREATIC EVENTS THAT LEAD TO THE DEVELOPMENT OF IDDM IN THE BB/E RAT.



DISCUSSION

In this study, serial pancreatic biopsy was used to further document the sequence of pancreatic events leading to the development of IDDM in the BB/E rat, and to examine the effect of CSA administered for a limited time in the prediabetic period on the events, and on the development of IDDM in DP rats held throughout their natural life span. None of the previous reports on the effects of CSA given in the prediabetic period (426-430, 729, 730) have involved such an extended follow-up, and the variable results obtained in these studies may in part be related to the differences in the period of observation following treatment.

The complete prevention of diabetes by CSA in BB/E rats aged less than 150 days is in agreement with the initial study by Laupacis et al (427) in which there was no subsequent follow-up. However, our study showed that cessation of CSA treatment was followed by the development of IDDM in 37% of the rats, although the frequency of diabetes was still considerably lower than that of the oil treated controls. Similar findings have been reported by other groups (429, 729, 730).

Thus, complete prevention of IDDM after administration of CSA from 34-121 days of age, was followed by the onset of diabetes in 24% of animals at 169 days of age, after withdrawal of CSA (729). Equally, a reduced incidence of diabetes and delayed onset was reported by Brayman et al (730) using a protocol of intermittent CSA therapy. They administered high doses (15mg/kg/day) of CSA initially for 2 weeks commencing between 30-49 or 50-55 days of age, followed by a similar twice weekly maintenance dose until 160 days, which resulted in low trough plasma CSA levels. Although 9 animals developed diabetes during CSA treatment, and a further 14 rats followed until 275 days of age subsequently became

diabetic, the overall incidence of diabetes was approximately half that of the controls (27% \leq 48%).

Using a limited protocol, whereby CSA was administered only for 10 day periods at different ages, Like et al (429) also delayed the onset and reduced the frequency of diabetes in DP BB rats. Although follow-up was limited to 120 days of age, CSA administered between 60-70 days of age decreased the incidence of diabetes to 23% compared with 73% in controls. However, similar administration of CSA either before 60 days or after 70 days had no effect on the incidence of diabetes thereby suggesting that the presence of CSA only at a critical stage in the prediabetic period may induce long-term prevention.

The observations from our study do not agree with other reports, demonstrating either the complete prevention of diabetes by CSA (428) or its lack of effect in reducing the development of glycosuria (430). In the former study, prevention of IDDM was achieved only by sustained treatment (from 5 to 25 weeks of age) with higher doses of CSA (10-20mg/kg/day). In addition, the follow-up period did not exceed 150 days post withdrawal of CSA. Furthermore, this protocol also prohibited the development of thyroiditis and gastritis and therefore differs from the results of Like et al (429) where CSA failed to protect against the development of lymphocytic thyroiditis.

In the study by Yale et al (430), although CSA did not affect the incidence of hyperglycaemia, it did however result in a milder form of diabetes with no weight loss, persistence of islet beta cells and only intermittent insulin requirements. Indeed, the observed diabetic syndrome was similar to the hypoinsulinaemic glucose intolerance induced in DR BB rats given CSA (694).

However, it is noted that in their study, the incidence of diabetes among the controls was only 29% i.e. still less than the incidence among our CSA treated animals, and this low spontaneous frequency may contribute to their failure to detect an effect of CSA on the incidence of diabetes (430).

Our findings that CSA administered at disease onset, along with insulin, failed to induce remission of diabetes or even lower the insulin requirements necessary to maintain normoglycaemia, are in agreement with other studies. Yale et al (732) found that CSA (10mg/kg/day) administered to 10 diabetic rats of recent onset, had no beneficial effect, as their daily insulin requirements over a 2 month period were identical to those of the controls. Equally, in an earlier study, where CSA was used as part of a multiple drug therapy, administration of CSA at disease onset only induced remission in one (2%) animal (426). This contrasted with a 22% cure rate when animals were given methylprednisolone combined with 3 injections of rabbit antiserum to rat lymphocytes. In addition, although combined treatment of CSA and a MoAb against the IL-2 receptor normalized the plasma glucose levels of BB rats with mild hyperglycaemia, neither CSA nor IL-2R MoAb on their own had any effect (733).

Taken together the results suggest the necessity of a functional beta cell reserve for successful immunotherapy of IDDM in the BB rat. This is supported by the results from CSA trials in human IDDM patients, where the major differences between those patients with remission and those without, include: the shorter duration of symptoms before diagnosis; the lesser degree of weight loss; lower initial haemoglobin A1c levels; higher initial C-peptide concentrations and lower frequency of ketoacidosis (734). Furthermore,

pancreatic biopsies taken from BB/E rats at onset of diabetes when stained for insulin, showed that many of the islets were insulin deficient. After disease onset the autoimmune destruction of surviving beta cells continued, such that biopsies removed 50 days after onset, with the animals having been maintained on insulin or insulin and CSA, in most cases showed a complete absence of insulin-containing cells.

Direct inhibitory effects of CSA on pancreatic insulin release have been reported both in vitro (735) and in vivo, where impaired glucose tolerance in normal Wistar (736) and DR BB (694) rats ensues. Electron microscopy of islets from normal Wistar rats, treated with 40mg/kg/day for 7 days showed severe degranulation, cytoplasmic vacuolization and dilation of endoplasmic reticulum in the beta cells (737). In addition, islets isolated from CSA treated animals showed a 50% reduction in mRNA (737). In our study, plasma concentrations of glucose and its gluconeogenic substrates, lactate and alanine, appeared elevated, although only the glucose levels reached statistical significance. These changes were reversible and not associated with the increase in lipolysis and rise in glycerol and 3-hydroxybutyrate levels, normally associated with onset of diabetes in BB/E rats (309). Thus, in addition to its immunosuppressive properties, CSA may have toxic effects on pancreatic beta cells, and this may contribute to the surprising observation that CSA administered to DR BB rats from 45-155 days of age, induced the development of diabetes in 5 of 16 (31%) animals (738).

The necessity to administer CSA in an olive oil vehicle due to its hydrophobic nature (696), resulted in elevated plasma triglycerides of comparable levels in both CSA treated and control groups. Thus, CSA per se did not affect plasma triglycerides, and nonetheless,

the levels returned to those of untreated controls shortly after withdrawal of treatment.

Administration of CSA in organ transplantation has been associated with several side effects of which nephrotoxicity is the most common and most severe (739). Nephrotoxicity induced by CSA therapy can be divided into two major groups: (i) functional toxicity without morphological lesions, and (ii) morphologic toxicity with tubular and/or vascular-interstitial lesions (740). In our study, CSA therapy did not induce functional nephrotoxicity as measured by plasma creatinine levels. This is in agreement with Brayman et al (730) who observed no difference in serum creatinine levels between CSA treated DP BB rats and controls. Furthermore, they and Laupacis et al (427) found no histologic evidence of vasculopathy or tubulointerstitial disease following treatment with CSA. However, Yale et al (732) reported a 50% increase in plasma creatinine after 5 weeks of CSA therapy when given at disease onset, and suggested that CSA induced nephrotoxicity may be enhanced in the presence of hyperglycaemia.

Concerning other potential side effects, in agreement with the early studies of Borel et al (699), we failed to observe any deleterious effect of CSA on peripheral blood leucocyte numbers suggesting that CSA does not exert its immunosuppressive actions by eliminating clones of cells. This is supported by the observation that IDDM still developed in some animals after withdrawal of CSA.

Examination of pancreatic biopsies from oil treated and CSA treated animals, was aimed to further document the sequence of pancreatic events that lead to IDDM in the

BB/E rat, and determine the effect(s) of CSA on these events.

Analysis of biopsies from oil treated controls further substantiated the findings of our previous biopsy study; which I have described in Chapter 6. Amongst the biopsies removed from CSA treated animals during treatment, the only abnormality observed was enhancement of islet MHC class I expression. Thus, in some animals, CSA was unable to block the increase in intra-islet class I expression, but in all animals prevented subsequent pancreatic infiltration and insulinitis. As mentioned previously, the cause of this increased islet class I expression is unknown, but release of viral induced interferons within the islets has been suggested (593). Thus, although CSA can inhibit the synthesis of IFN-gamma by human T lymphocytes (741), the report that demonstrates its failure to inhibit production of IFN-alpha/beta by virus-infected human or mouse lymphocytes and fibroblasts (742) lends support to this postulate and is in keeping with our in vivo observations.

Two different models are generally proposed to explain the biological activity of CSA (743). The first proposes that different T cell subsets show differing sensitivities to CSA. Accordingly, CSA can inhibit the activation of T_H cells, and since "help" is needed to activate the cytotoxic subset, it consequently inhibits the generation of T cyt cells (701). Equally, the model postulates that T supp cells are resistant to the actions of CSA (702, 703). Thus, the observation that autologous splenic lymphoid cells from "not-yet" diabetic NOD mice, when cultured for 72h with CSA and IL-2 before reinfusion into the animal from which they were isolated, prevented the development of diabetes in 68% of the animals, is suggestive of an ex vivo preferential IL-2 activation of specific suppressor

cells for the autoimmune process, with CSA blockade of the helper/cytolytic activities (744).

The second model does not distinguish between the activities of subsets as defined by antigenic markers. This model proposes that CSA interrupts the transmission of the antigen-specific signal from the surface to the interior of the cell after binding of antigen and T cell receptor (745). This is supported by the observation that whereas antigen or mitogen induced lymphokine release from activated T cells is inhibited by CSA, it does not inhibit binding of antigen to the T cells (713). Equally, constitutive lymphokine production by tumour cell lines is unaffected by CSA (709), and once T cells have been activated to produce mRNA for lymphokine synthesis, they become insensitive to CSA (712). Therefore, CSA does not block the translation of lymphokine encoded message once it has been formed.

Thus in the main, CSA is believed to exert its immunosuppressive effect by inhibiting the production of mRNA for lymphokine production by primed T cells. However, in our study, the most striking feature in biopsies from CSA treated animals was the prevention of the initial accumulation of ED1⁺ cells at perivascular or periductal sites adjacent to islets free from infiltration, thereby suggesting a direct inhibitory effect of CSA on macrophage recruitment. It cannot be completely excluded that this apparently direct effect of CSA on macrophages is not secondary to a primary action of CSA in blocking the function of primed T cells. Nevertheless, T lymphocytes were certainly not conspicuous in any of the biopsies examined during treatment, and other studies in the BB rat (589) and the low-dose streptozotocin model (508) have indicated that T cell infiltration is macrophage dependent.

Certain macrophage functions, including phagocytosis of bacteria (719), blood clearance of colloidal carbon (746) and migration in response to lymphokines (714), are all reportedly unaffected by CSA. However, other studies indicated that CSA impaired monocyte/macrophage IL-1 production (701) and chemotaxis (747). Furthermore, several studies have demonstrated that CSA inhibits monocyte accessory functions in oxidation - (748), mitogen - (749), alloantigen - (750), and antigen - dependent T cell proliferation assays (751-754).

Varey et al (752) reported that CSA affected the ability of irradiated mouse spleen cells to present preprocessed antigen to mouse thyroglobulin and purified protein derivative - specific T-cell lines. Similarly, Palay et al (753) and Snyder et al (754) demonstrated that pretreatment of macrophages with CSA resulted in a cell population with a markedly diminished capacity to support the activation of murine and human T cells respectively. Both studies showed that this effect of CSA on antigen presentation was unrelated to decreases in IL-1 production, increases in prostaglandin production or changes in the levels of surface Ia expression. In contrast, Whisler et al (750) found that the impaired ability of human macrophages to trigger mixed lymphocyte responses following pre-exposure to CSA, was associated with a decrease in macrophage HLA-DR expression. Palay et al (753) suggest that the hydrophobic nature of CSA permits it to insert into the phospholipid bilayer of plasma membranes where it may destabilize the complexing of processed antigen with Ia molecules on the surface membrane, such that the T cell receptor cannot bind them and thus become activated.

Therefore, in addition to its extensively documented effects on T lymphocyte activities, CSA can also directly inhibit certain macrophage accessory functions.

Thus, since macrophages and other accessory cells play a critical role in the activation of certain T cell responses (578), the immunosuppressive actions of CSA may be explained in part by its inhibitory effect on the induction of immune responses by macrophages.

However it should be stressed that most of the studies aimed at investigating the mechanisms of CSA immunosuppression have been performed *in vitro*, and it is possible that the mode of CSA action is quite different *in vivo* (755). Accordingly, Krocze et al (756) reported that the administration of CSA *in vivo* had no effect on alloantigen-induced increases in cell size, percentage of cells expressing the IL-2 receptor, the spontaneous or IL-2 driven proliferation of freshly explanted cells, or the induction of cytotoxic T lymphocyte activity.

In addition to the absence of macrophage infiltration, biopsies from CSA treated animals also failed to show the accompanying expression of MHC class II molecules on the vascular endothelium. Such an observation is compatible with the demonstration that CSA inhibits Ia expression on ocular tissues in guinea pigs with experimental autoimmune uveitis (757). Equally CSA has been shown to inhibit lymphokine-induced class II MHC expression on vascular endothelium both *in vitro* (758) and *in vivo* (759).

In conclusion, CSA administered from 30-100 days of age completely prevented the development of IDDM up to 150 days of age and reduced the incidence of diabetes to approximately 50% of controls at 450 days. Whereas ICSC paralleled the development of diabetes, IAA were unrelated to diabetes onset and unaffected by CSA. Analysis of pancreatic biopsies showed that CSA had no effect on pancreatic hyperexpression of MHC class I

molecules but markedly inhibited accumulation of ED1⁺ cells at extra islet sites, the subsequent recruitment of other immune effector cells and islet infiltration. This resulted in a delay on the onset of diabetes in some animals and prevention of diabetes in others. Commencement of CSA therapy at disease onset failed to ameliorate the disease and is compatible with the view that once effector cells have been activated, CSA has little or no effect.

The reason why treatment with CSA prevented the development of IDDM in some animals, whilst merely delaying its onset in others, is not clear. It possibly reflects individual animal variation in the stage of disease development at the time of CSA treatment. This is supported by the study of Like et al (429) which showed that only when CSA was administered between 60-70 days of age was a protective effect observed, thereby suggesting a "critical window" for intervention. Thus, it is possible that administration of CSA for a limited period, started at a critical point in the autoimmune process, could lead to long term protection. However the difficulty would be in establishing this critical period in individual subjects.

Meanwhile, ongoing clinical trials suggest that whilst CSA can induce remission in some recently diagnosed IDDM patients, maintenance of this remission probably necessitates continual long term CSA therapy. These trials have not been running sufficiently long enough to ascertain the possible hazards of long term CSA therapy. However, in addition to nephrotoxicity, evidence from organ transplantation centres, where CSA has been used to prevent graft reject since 1977, indicate an increased risk for developing cancers (760) which in turn may arise from an increased susceptibility to viral infections (761). Whereas the average time of cancer

appearance in patients receiving conventional immunosuppressive therapy (CIT) (azathioprine and prednisolone) was 68 months, cancers that appeared after CSA therapy occurred at an average of only 26 months after transplantation (760). In addition, analysis of data from the Cincinnati Transplant Tumour Registry shows a disproportionately high incidence of lymphomas (27% v 11%) and Kaposi's sarcoma (11% v 3%) in CSA patients compared with CIT patients (760).

Thus, until such time that an accurate early predictive marker for the disease becomes available, at which point short term intermittent CSA therapy may be applicable and provide long term prevention, I would not favour the use of CSA in the treatment of patients with IDDM.

CHAPTER 9 - SUMMARY AND CONCLUDING REMARKS

TABLE XVII: COMPARISON OF HUMAN IDDM AND ITS ANIMAL MODELS

Characteristics	Human	BB	NOD	LD-STZ
<u>Clinical Features</u>				
Spontaneous onset of IDDM	+	+	+	-
Insulin dependence	+	+	+	-
Proneness to ketoacidosis	+	+	+	-
Lean body habitus	+	+	+	+
Equal sex distribution	+	+	-	-
Association with other endocrinopathies	+	+	+	-
Evidence of diabetic complications	+	+	+/-	-
<u>Morphology</u>				
<u>Insulinitis:</u>				
$\text{M}\phi$	+/-	+	+	+
T_H cell	+	+	+	+
T cyt/supp cell	+	+	+	+
B cell	+	+	+	+
NK cell	?	+	+	+
Infiltration in other tissues	+	+	+	-
<u>Altered islet MHC antigen expression</u>				
Increased class I expression (without insulinitis)	+	+	?	-
Aberrant class II expression (without insulinitis)	+	-	+/-	-
<u>Humoral immunity</u>				
ICA	+	-	+	-
ICSA	+	+	+	+
IAA	+	+	+	-
CF-ICA	+	+	?	-
Anti-64KD	+	+	?	-
Autoantibodies to other tissues	+	+	+	-
<u>Cellular immunity</u>				
Association with MHC genes	+	+	+	+
Increased levels of circulating Ia^+ T cells	+	+	?	-
Demonstration of passive transfer	+/-	+	+	+/-
Demonstration of circulating beta cytotoxic cells	+	+	+	+
Disease prevention or reversal by immunosuppression	+	+	+	+
Disease recurrence in islet transplants	+	+	+	+/-

In this thesis I have attempted to promote the relevance of the BB rat as a model for human IDDM. Table XVII summarizes some of the characteristics of human IDDM and its animal models, and clearly shows that most of the individual features of human IDDM are also observed in the BB rat. The results from our studies in the BB/E rat, supplement many other studies which also document the presence of immunological abnormalities in the BB rat. However, the mere presence of certain immunological phenomena, does not necessarily imply that IDDM in the BB rat is an autoimmune disease. Nevertheless, when considering Witebsky's postulates for determining the relationship of immunological phenomena to disease aetiology (22), there is strong support that IDDM in the BB rat is of autoimmune aetiology:

- (i) The autoimmune response is regularly associated with the disease.

Although the development of insulinitis does not always lead to the onset of diabetes, nevertheless, diabetes is invariably associated with the characteristic histologic appearance of insulinitis. In addition, the presence of antibodies against islet cell antigens, e.g. ICSA, are commonly associated with the development of the disease.

- (ii) A replica of the disease should be inducible in experimental animals

Although the spontaneously diabetic BB rat represents an "experimental" animal model of human IDDM, a replica of the disease in DP BB rats can be induced by immunomodulatory protocols on the DR BB rat. Thus, depletion of the RT-6⁺ T cell population in DR BB rats results in the development of IDDM (381).

- (iii) The animal develops pathological changes that are basically similar to those of the human.

Table XVII shows that most of the changes in pancreatic morphology associated with the development of IDDM in the human are also replicated in the BB rat. The two main differences are: (i) expression of MHC class II antigens on pancreatic beta cells is never observed in the absence of insulitis in the BB rat, but has been observed in infiltrate free islets in man (549), (ii) macrophages have not been reported to be a prominent feature of the infiltrate of the human pancreata examined (10% of infiltrates - Alan Foulis, personal communication), whereas the majority of cells infiltrating the islets at all stages of disease development in the BB rat are macrophages.

- (iv) The experimental disease should be transferable to non-immunized animals by serum or lymphoid cells.

Initially, transfer of diabetes into young (30-40 days old) DP BB rats was reported using ConA activated spleen cells from acutely diabetic BB rats (405). Subsequently, transfer of diabetes into immunosuppressed MHC-compatible WF rats and DR BB rats as well as BB hybrid rats (containing at least one RT1^u allele) was demonstrated (406,407). Furthermore, conditioned media, using ConA activated spleen cells from acutely diabetic rats, DR BB rats, WF rats and MHC-incompatible Buf rats was able to induce diabetes in both DP and DR BB rats but not WF rats (410).

In addition to the above criteria, the ability to prevent or ameliorate the disease by manipulation of the immune system via immunosuppression with cyclosporine or reconstitution of the immune system by bone marrow transplantation (416), or spleen cell transfusions (422), further substantiates the concept of an autoimmune aetiology for IDDM in the BB rat.

The demonstration of passive transfer of diabetes in both DP and DR BB rats using conditioned media from BB and non-BB rat strains (410) suggests that a soluble factor obtained from normal splenocytes can activate an effector population in both DP and DR BB rats. Equally, the development of IDDM in DR BB rats following depletion of the RT6⁺ T cell population (381) suggests that RT6⁺ lymphocytes may regulate beta cell cytotoxic effector cells in the DR BB rat to prevent the development of diabetes in these animals.

Thus in the BB rat it is postulated that expression of diabetes depends on the balance between beta cytotoxic effector cells and regulatory cells. In both the DP and DR BB rats, "forbidden clones" of autoreactive lymphocytes specific for beta cell antigens or cross reactive antigens are part of the normal immune repertoire. Accordingly, it is not uncommon to find evidence of insulitis and accompanying beta cell destruction in some DR as well as DP BB rats. However, in the DR BB rat, as well as some of the DP BB rats (i.e. the 30-40% who do not develop IDDM), it is envisaged that a regulatory population of lymphocytes (possibly T suppressor cells) either acting directly or through the idiotypic network can restrict the autoaggressive immune response to prevent the onset of IDDM. Other factors which may influence the "susceptibility" or "resistance" to diabetes include the ability to increase beta cell replication and thereby

partly counteract the forces of beta cell aggression. Equally, differences in the strength of the autoimmune assault within individual animals may determine whether or not they succumb to the disease.

Recently, Molvig et al (762) reported that TNF-alpha secretion by isolated human monocytes stimulated by LPS show marked interindividual differences. Since TNF-alpha can mediate islet cell cytotoxicity and potentiate both IL-1 (412) and IFN-gamma (670) mediated islet cell damage, Nerup et al (763) have proposed that "quantitative" differences in the magnitude of the autoimmune response propagated by a high TNF-alpha secretor phenotype may contribute to the "susceptibility" of individuals for developing IDDM.

Since the genes encoding for TNF-alpha in both man (764) and mouse (765) are linked to the MHC genes on chromosome 6 and 17 respectively, it is suggested that the MHC associated genetic susceptibility for IDDM reflects linkage disequilibrium between MHC class II genes and the TNF-alpha gene (763).

Although the genetic susceptibility for IDDM is strongly associated with class II MHC genotypes in both man (146) and the BB rat (338), the susceptibility is not due to unique or mutant class II alleles in either species. Whereas the presence of a non charged amino acid (ser, val, ala) at position 57 of the first domain of the DQ beta chain is positively associated with the development of IDDM in Caucasians, the possession of Asp 57-positive DQ beta alleles provides resistance (177). However, IDDM in Japanese and Chinese patients is associated with the DQ beta 3.3 chain which has an aspartic acid residue at position 57 thus suggesting that other amino acid residues contribute to susceptibility (766).

In the BB rat, although both the RT1B beta and RT1D beta chains possessed a serine residue at position 57, the class II beta chains were identical in both DP and DR BB rats which led the authors to suggest that the allelic form of amino acid 57 is not a disease susceptibility marker in the BB rat (341). Nevertheless, since both DP and DR BB rats harbour autoreactive clones with specificity for beta cell antigens and both sublines can develop insulinitis, it is possible that the beta chain sequences of the MHC class II genes in the BB rat do confer an increased susceptibility for IDDM, but subsequent manifestation of the disease depends on other genes perhaps in linkage disequilibrium with the class II MHC genes. Thus, it is unlikely that one specific allele determines disease susceptibility and therefore assessment of MHC disease association must consider the extended MHC haplotype.

The aim of the studies in this thesis was to better understand the aetiology of IDDM in the BB rat. The use of serial pancreatic biopsy within individual BB/E rats and MoAbs against rat MHC antigens, lymphocyte and macrophage subsets has enabled the documentation of the sequence of pancreatic events that lead to development of IDDM in the BB/E rat.

The first change observed; increased expression of MHC class I antigens within some islets and on vascular endothelium, could be observed at least 30 days before the onset of diabetes. Between 2 and 3 weeks before disease onset, expression of MHC class II molecules on vascular endothelium is associated with the emigration from the circulation of a population of macrophages into the pancreatic tissue. Subsequent migration of these recruited macrophages into the adjacent islets soon follows, thereby initiating insulinitis.

Release of cytokines e.g. IL-1 and TNF-alpha by these activated macrophages may directly injure the islet beta cells, but can also influence the production of chemotactic factors, synthesis of adhesion molecules, and vasodilation of the nearby endothelial cells (767) thereby aiding the subsequent recruitment of other leucocytes. Thus by 10 days before onset of diabetes, insulinitis comprising macrophages, T-cells, B-cells and NK cells is observed in most of the islets. At this stage, hyperexpression of MHC class I molecules within infiltrated islets is markedly enhanced, extending beyond the islet periphery into the surrounding exocrine tissue. "Aberrant" expression of MHC class II molecules on insulin-containing cells was a rare phenomenon and was never observed in the absence of insulinitis.

Within a week of onset of diabetes, ketoacidosis will develop unless insulin is administered. In biopsies from both ketoacidotic and recently diagnosed but insulin treated animals, virtually all of the islets are "end-stage", devoid of insulin-containing cells, insulinitis and enhanced MHC class I antigen expression. Thus the use of serial pancreatic biopsy has enabled us to perform time course studies in individual animals where changes in pancreatic morphology have been correlated with the development of IDDM. Nevertheless, our studies have still left several important questions unanswered:

(i) What initiates the autoimmune response?

The proposal that aberrant expression of MHC class II antigens on pancreatic beta cells may permit presentation of beta cell antigens to potentially autoreactive T lymphocytes (540), was not supported by our studies. Since "classical" antigen presenting cells such as macrophages are prominent among the insulinitis and indeed appear

to be the first cells which infiltrate the islets, it can be postulated that presentation of beta cell antigens by macrophages to autoreactive T_H cells will propagate the autoimmune response. Thus any agent which can perturb the islet beta cell and result in the release of sequestered antigen(s) can lead to the development of the autoimmune response if the antigen(s) is taken up, processed and presented to the appropriate "forbidden clones". Thus, no specific aetiological agent is necessary but rather anything e.g. beta cytotropic viruses, dietary toxins, metabolic stress, or increased systemic IL-1/TNF concentrations, which can damage the islet beta cells, may trigger the autoimmune response. Equally, the studies on the DR BB rat suggest that any process e.g. immunosuppression which disturbs the balance between effector and regulatory cell populations may also trigger the autoimmune response.

(ii) What cell(s) mediate beta cell destruction?

Within the insulinitis, there are a variety of different leucocytes including macrophages, T-lymphocytes (both $CD4^+$ and $CD8^+$), NK cells, and B-lymphocytes, however it is not known which of these cells mediate beta cell damage and which are merely "innocent bystanders". Evidence suggests that islet cell destruction is non-MHC-restricted (397) which may favour a role for NK cells, however both $CD4^+$ and $CD8^+$ T cell populations can also initiate MHC-unrestricted killing of target cells (768) and T cells with MHC-unrestricted cytotoxic function have been found in inflammatory rheumatoid synovial fluid (769). In the NOD mouse, cloned T cells specific for islet beta cells can be either $CD4^+$ or $CD8^+$,

however diabetes can only be transferred to naive NOD mice using both the CD4⁺ and CD8⁺ clones (770). It is therefore likely that several different lymphocyte clones are involved in beta cell destruction.

This is supported by a recent study which demonstrated the prevention of diabetes in NOD mice following treatment with an anti-V beta 8 TCR MoAb (771). However, examination of the insulinitis lesions showed that not all the cells were V beta 8⁺ and in treated animals despite the absence of V beta 8⁺ T cells, diabetes still developed in some animals.

(iii) What is the mechanism of beta cell destruction?

In vitro studies have demonstrated that the cytokines IL-1, TNF and IFN-gamma either singly or more potently in combination can mediate beta cell cytotoxicity (412, 413, 670). Since these cytokines can be synthesized by either macrophages or T-lymphocytes both of which are present in the insulinitis, it is possible that these cytokines are present in high local concentrations within infiltrated islets, where they can cause beta cell cytolysis. Amongst the proposed mechanisms for cell killing by cytokines; the generation of oxygen free radicals in the target cell coupled with a low oxygen free radical scavenging enzyme activity for beta cells, is favoured. This was supported by a recent study which demonstrated that oxygen free radical scavengers could protect rat islet cell monolayers from the cytotoxic effects of the above mentioned cytokines (772).

In conclusion: Although replacement therapy has been available for Type 1 diabetes mellitus for almost 70 years, treatment with insulin remains unsatisfactory as it is still associated with increased morbidity and mortality. This stresses the importance of research into the aetiological factors of the disease, with the aim of developing protocols leading to disease prevention. However, the development of a rational therapeutic approach to prevention necessitates the identification of accurate markers for the development of clinical disease as distinct from genetic susceptibility, and elucidation of the precise sequence of events that lead to the destruction of islet beta cells. Difficulties in correlating changes in cellular and humoral immunity with pancreatic morphology and function in man, as well as ethical problems in the application of certain intervention studies, necessitates the need for studies using animal models.

In this thesis, I have hopefully shown that diabetes in the BB rat can be considered of autoimmune aetiology, and that the diabetic syndrome of the spontaneously diabetic BB rat closely resembles the human disease. Without the help of animal models, patients with IDDM would still be dying in ketoacidosis. With the help of animal models such as the BB rat, the possibility of predicting and preventing IDDM is not unrealistic.

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APPENDIX

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Rapid communications

Pre-diabetes in the spontaneously diabetic BB/E rat: lymphocyte subpopulations in the pancreatic infiltrate and expression of rat MHC class II molecules in endocrine cells

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Summary. Use of monoclonal antibodies specific for rat lymphocyte subsets and an anti-insulin marker has allowed us to document the following sequence of events leading to the development of clinical diabetes in this animal model. The first change observed in the pancreas is increased expression of MHC class II molecules on vascular endothelium and this precedes lymphocytic infiltration. Next, T cells of the T helper phenotype infiltrate the pancreas around blood vessels. Many of the infiltrating T cells show class II expression indicating that they are activated. A few cytotoxic and suppressor cells

and B lymphocytes are also present and their numbers increase proportionately with rat age. Some macrophages are also seen. Finally, at a late stage class II MHC molecules can be detected in partially destroyed islets on β cells which are still actively synthesising insulin. We have never observed expression of class II molecules on glucagon or somatostatin secreting cells which are invariably well preserved.

Key words: Pre-diabetes, BB rat, lymphocyte subsets, β cell, MHC class II expression.

The BB rat is probably the best available animal model of human Type 1 (insulin dependent) diabetes mellitus [1]. In these animals islet cell surface antibodies and a mononuclear cell pancreatic infiltrate precede the development of the disease which has been shown to be associated with the RTI^a haplotype of the rat MHC [2].

As part of a longitudinal study designed to show the relationship between autoimmunity markers, islet morphology and β -cell function [3] we have examined the distribution of subpopulations of lymphocytes infiltrating the pancreas. Since there is some evidence in organ specific autoimmune states that affected glands aberrantly express class II molecules [4, 5] the pancreas were also extensively screened for the presence of Ia molecules on endocrine cells.

Materials and methods

Animals

The animals used in this study were from the Edinburgh colony (BB/E), the nucleus of which was kindly donated in 1982 by Dr P. Thibert, Animal Resources Division of Canada, Ottawa. The average incidence of diabetes in this colony is 60–70% and the mean age at onset ~96 days. A cohort of 96 BB/E rats was studied prospectively with eight male and eight female normoglycaemic rats being selected for study every 15 days between 30 and 105 days of age.

Protocol

At each time point, selected rats received an IP injection of ³H thymidine (0.5 μ Ci/g body weight) 1 h before being killed under light halothane anaesthesia. The pancreases were rapidly excised and serum samples were taken and stored at -20°C for determination of glucose and insulin concentrations and autoantibodies to thyroglobulin, gastric parietal cells, smooth muscle, islet cell surface and cytoplasm and insulin. Pancreatic tissue was divided into three portions which were either (a) immediately snap-frozen in isopentane at -70°C for examination of lymphocytic infiltration and expression of class II rat MHC molecules, (b) fixed in Bouin's solution for autoradiography, or (c) used to prepare isolated islets for insulin secretion studies. This report describes only the observations relating to lymphocytic infiltration and class II MHC antigen expression on frozen sections.

Antisera

The following monoclonal antibodies were generously provided by Dr. D. Mason, Sir William Dunn School of Pathology, Oxford. OX6, specific for rat Ia; W3/25 which recognises T helper (T_H) cells and macrophages; OX8, a T cytotoxic/suppressor (T_c/s) cell marker and OX19 (pan T). B lymphocytes were recognised using OX12 which is specific for rat Kappa chains. Novo, Copenhagen, supplied the following monoclonals specific for endocrine cells: anti-human insulin (HU1 018); anti-glucagon (GLU 001) and anti-somatostatin (SOM 018) and rabbit anti-glucagon sera and a guinea pig antibody (M1183) used to detect insulin containing cells. Rabbit anti-somatostatin sera was generously provided by Dr. Patel, Montreal.

Immunofluorescence

Serial sections (4 μ m) of snap-frozen pancreas were scanned for lymphocytic infiltration, endocrine cells and aberrant Ia expression using the above monoclonal antibodies. Clone supernatants were used at

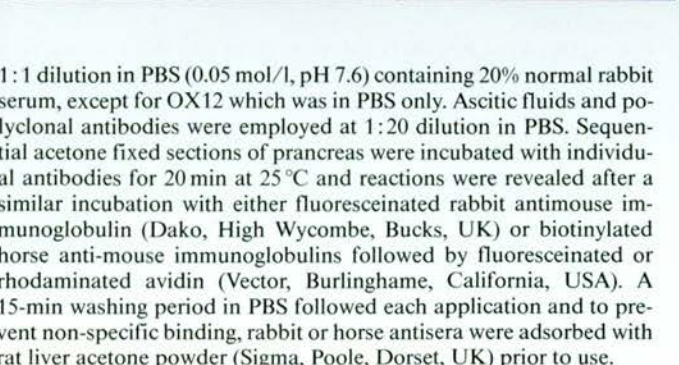
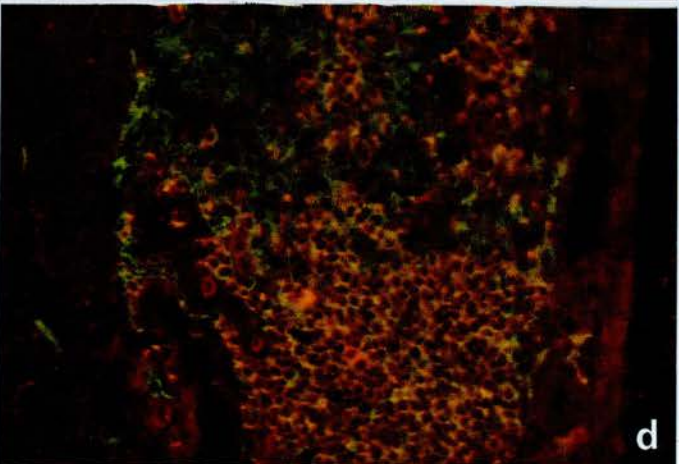
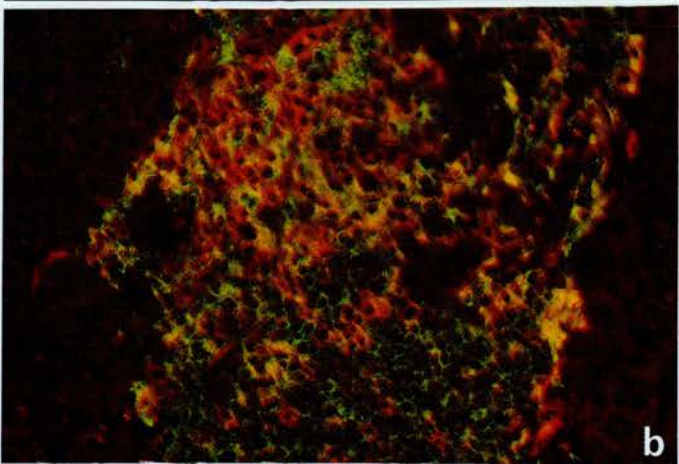
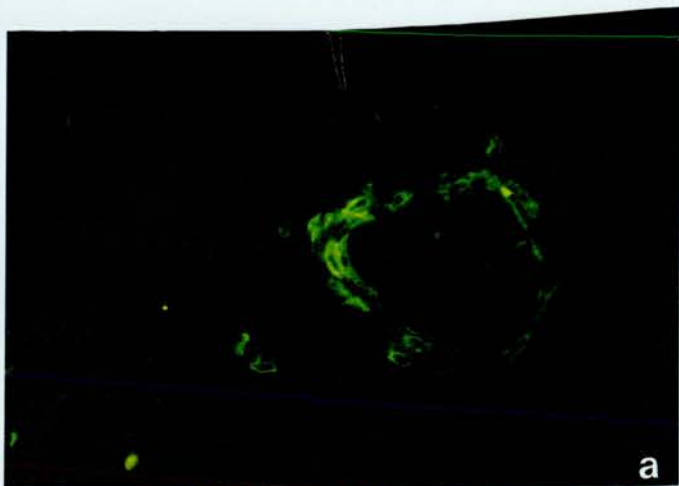
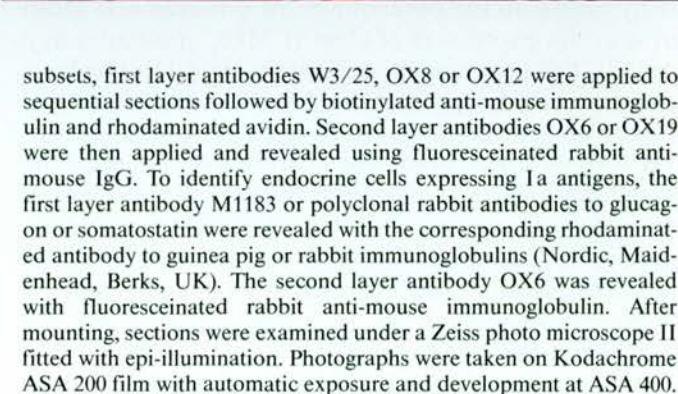
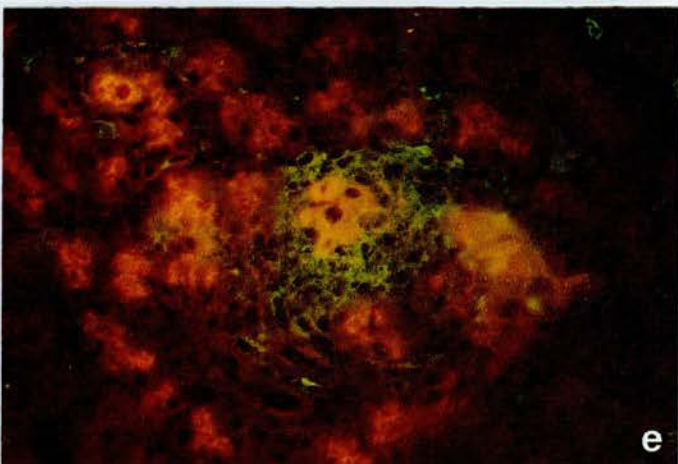
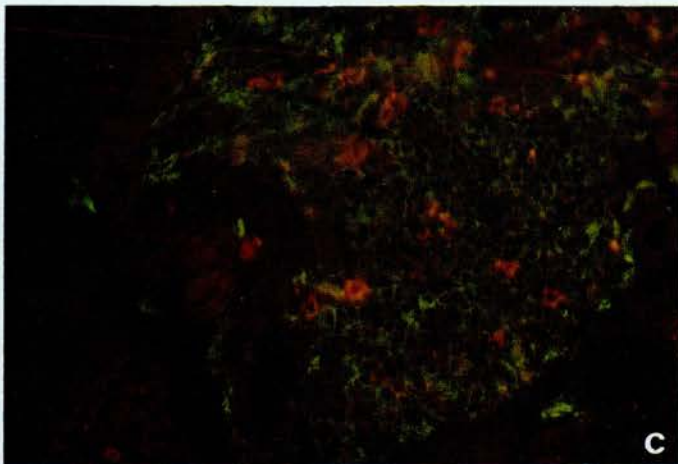


Fig. 1a-e. Cryostat sections of pre-diabetic BB/E rat pancreas stained by immunofluorescence using antibodies as described in Materials and Methods. **a** OX6 revealing Ia expression on vascular endothelium. Rat age 45 days ($\times 640$) **b-e** Double exposure photographs taken successively using normal green and red filters of the Zeiss photomicroscope II. **b-d** Sequential sections from a 105-day-old rat ($\times 400$): **b** W3/25 (anti-T helper and some macrophages) and OX6 (anti-Ia). **c** OX8 (anti-T cytotoxic/suppressor) and OX6. **d** OX12 (anti-immunoglobulin Kappa chain) and OX6. **e** M1183 (guinea pig antibody reacting with pro-insulin) and OX6 clearly show the expression of class II molecules on some insulin-producing cells. Rat age 75 days ($\times 640$)



1:1 dilution in PBS (0.05 mol/l, pH 7.6) containing 20% normal rabbit serum, except for OX12 which was in PBS only. Ascitic fluids and polyclonal antibodies were employed at 1:20 dilution in PBS. Sequential acetone fixed sections of pancreas were incubated with individual antibodies for 20 min at 25°C and reactions were revealed after a similar incubation with either fluoresceinated rabbit antimouse immunoglobulin (Dako, High Wycombe, Bucks, UK) or biotinylated horse anti-mouse immunoglobulins followed by fluoresceinated or rhodaminated avidin (Vector, Burlingame, California, USA). A 15-min washing period in PBS followed each application and to prevent non-specific binding, rabbit or horse antisera were adsorbed with rat liver acetone powder (Sigma, Poole, Dorset, UK) prior to use.

In double fluorochrome experiments to recognise lymphocyte

subsets, first layer antibodies W3/25, OX8 or OX12 were applied to sequential sections followed by biotinylated anti-mouse immunoglobulin and rhodaminated avidin. Second layer antibodies OX6 or OX19 were then applied and revealed using fluoresceinated rabbit anti-mouse IgG. To identify endocrine cells expressing Ia antigens, the first layer antibody M1183 or polyclonal rabbit antibodies to glucagon or somatostatin were revealed with the corresponding rhodaminated antibody to guinea pig or rabbit immunoglobulins (Nordic, Maidenhead, Berks, UK). The second layer antibody OX6 was revealed with fluoresceinated rabbit anti-mouse immunoglobulin. After mounting, sections were examined under a Zeiss photo microscope II fitted with epi-illumination. Photographs were taken on Kodachrome ASA 200 film with automatic exposure and development at ASA 400.

Results and discussion

This preliminary paper is based on scanning studies using at least 8 pancreases at each time point. The first change observed was an increased expression of class II molecules occurring on what appeared to be vascular endothelium (Fig. 1a); This preceded lymphocytic infiltration of the gland, which was not apparent until 60 days of age in these animals. Insulitis was observed by scanning adjacent sections with monoclonal anti-Ia and it was confirmed using Bouin-fixed tissue stained with haematoxylin and eosin. Twenty five percent of the pancreases were infiltrated by 60 days and 62% by 105 days. These lymphocytic infiltrates, mainly consisting of cells which were OX19⁺ and W3/25⁺ appeared to emanate from the vessels and converge on islets. By double fluorochrome staining on sequential sections it was established that the majority of invading lymphocytes were also Ia⁺, suggesting recent activation (Fig. 1b). It has been shown that Ia expression on rat T cells is an early and perhaps transient indication of activation (P. Chisholm, personal communication).

A few isolated cells were double stained with W3/25 and OX6 and had a morphological resemblance to macrophages. The latter have been observed in pancreatic infiltrates by electron microscopy [6]. There is unfortunately as yet no marker in the rat for differentiating Tc from Ts, thus we are unable to determine whether the few OX8⁺ cells found in infiltrates represent mainly cytotoxic effectors or whether they are suppressor cells acting to dampen the immune response. Most of the infiltrating OX8⁺ cells were Ia⁻ (Fig. 1c).

B lymphocytes were identified in all pancreatic infiltrates, their numbers increasing proportionally with rat age. A few plasma cells could also be identified among the infiltrating B lymphocytes by their cytoplasmic immunoglobulin staining (Fig. 1d). It is interesting that T and B lymphocytes appeared to occupy distinct areas in the infiltrates.

Coincident with infiltration by activated lymphocytes, occasional endocrine-like cells were seen to express Ia. When sections were double stained with antibody to insulin, some β cells were identified containing insulin and expressing Ia (Fig. 1e). This is the first time this has been demonstrated in the BB rat and is strikingly similar to the observations of Bottazzo and Dean of aberrant expression of class II MHC molecules in β cells in the pancreas of a diabetic child [5]. We have never observed expression of class II molecules on glucagon or somatostatin secreting cells, which are invariably well preserved. The cytoplasmic nature of the β -cell staining with OX6 and lack of OX6⁺ glucagon or somatostatin secreting cells would seem to preclude passive acquisition of Ia molecules. However, proof of active synthesis of these antigens by insulin-producing cells can come only from studies employing *in situ* hybridisation for messenger RNA class II MHC probes. Ia⁺ positive β cells have been observed mainly in par-

tially disrupted islets. Several workers have shown either increased Ia expression or *de novo* Ia expression on cells exposed to γ interferon [7, 8] and it is not inconceivable that this or another lymphokine is being produced locally by the Ia⁺ T cells. In the prediabetic rat the finding of some islets with full insulin expression surrounded by Ia⁺ lymphocytes while the endocrine cells were Ia negative, suggests that β -cell Ia expression does not precede lymphocytic infiltration and may be a secondary rather than a primary phenomenon. The trigger initiating the whole process remains obscure.

Elucidation of the precise aetiology of Type 1 (insulin dependent) diabetes and detailed examination of the effects of possible therapeutic intervention is clearly difficult in man. This animal model offers the possibility of such studies.

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Rapid communication

Induction of class II MHC antigens in vitro on pancreatic B cells isolated from BB/E rats

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Summary. The ability of recombinant Interferon- γ to induce class II expression in vitro on pancreatic islet B cells has been investigated by exposing islets isolated from BB/E and normal Wistar rats to Interferon- γ and then staining successively with monoclonal antibodies specific for rat class II MHC antigens and insulin. Induction of class II expression was never observed on islet cells obtained from either normal Wistar rats

or rats from the BB/E low diabetes incidence (<2%) subline. In contrast, pancreatic B cells from rats from the BB/E high diabetes incidence (60–70%) subline expressed class II antigen following culture with Interferon- γ .

Key words: BB rat, diabetes, Interferon- γ , pancreatic B cell, MHC class II expression.

We have previously shown that aberrant expression of class II MHC antigens occurs on pancreatic B cells in the spontaneously diabetic, insulin-dependent BB/Edinburgh (BB/E) rat [1]; this phenomenon has been described also in a case report of human Type 1 (insulin-dependent) diabetes mellitus [2]. Neither the significance of this finding nor the mechanism by which it occurs is understood. Recombinant Interferon- γ (r IFN- γ) has been shown to induce class II MHC antigens on both rat (DC Rayner, HJ de Assis-Paiva, PM Lydyard, S Bidey, P van der Meide, A Cooke, unpublished observations) and human [3] thyroid epithelial cells in vitro but not on normal rodent [4] or human [5] pancreatic B cells. Susceptibility to autoimmune disease in both man and BB rats [6] is known to be linked to the MHC complex. We have therefore examined the ability of rat r IFN- γ to induce class II antigens on pancreatic B cells isolated from normal Wistar and BB/E rats.

Materials and methods

Animals

The animals used in this study were from the Edinburgh colony of BB rats, the nucleus of which was kindly donated by Dr. P. Thibert, Animal Resources Division of Canada, Ottawa in 1982. The colony now consists of two sublines created by selective breeding. In the high diabetes incidence line (BB/E/H) the incidence of insulin-dependent diabetes is 60–70% and the mean age at onset 96 days. In the low diabetes incidence line (BB/E/L) <2% of animals become diabetic by

120 days of age. BB/E/H animals were screened for circulating islet cell surface antibodies (ICSA) using a modified ¹²⁵I-protein A radio-ligand assay [7]. We have previously observed that over 90% of ICSA positive animals subsequently develop insulin-dependent diabetes (AJ Bone, unpublished observations). However, some ICSA negative animals also become overtly diabetic and require treatment with insulin.

Islet isolation, culture and dispersion

Islets were isolated by collagenase (Serva, Uniscience, London, UK) digestion from the following groups of animals, all of which were aged 80–90 days: 1) ICSA positive BB/E/H rats; 2) ICSA negative BB/E/H rats; 3) BB/E/L rats; 4) normal Wistar rats. For each experiment isolated islets were pooled from four rats in each animal group. Batches of pooled islets (350–400) were maintained in free-floating culture for 4–5 days in tissue culture medium (RPM1 1640, Imperial Laboratories, Salisbury, UK) containing glucose (11.1 mmol/l), HEPES (20 mmol/l), antibiotics (penicillin 100 U/ml; streptomycin 0.1 mg/ml), heat-inactivated 10% fetal bovine serum (Imperial Laboratories) with or without rat r IFN- γ (100 U/ml), a concentration we have previously established as optimal for Ia induction on cultured rat thyroid cells (DC Rayner et al., unpublished). The medium was changed after 2 days of culture. At the end of the culture period (4–5 days) islets were washed twice in ice-cold Ca²⁺ and Mg²⁺-free Hanks' Balanced Salt Solution containing penicillin and streptomycin (as above) and BSA (5 mg/ml) (HBSS-Ca²⁺/Mg²⁺). To prepare cell suspensions the washed islets were resuspended in HBSS-Ca²⁺/Mg²⁺ (1.5 ml) containing EGTA (2 mmol/l, Sigma Poole, Dorset, UK) and incubated for 20 min at 37°C in a shaking water bath to disperse the islet cells. Cell dispersion was completed by repeated (10 times) aspiration in a Pasteur pipette. The cell suspension was washed once in HBSS-Ca²⁺/Mg²⁺, once in RPM1 1640 (supplemented as above) and finally resuspended in RPM1 1640 (approximately 5 × 10⁵ cells/ml) for staining.

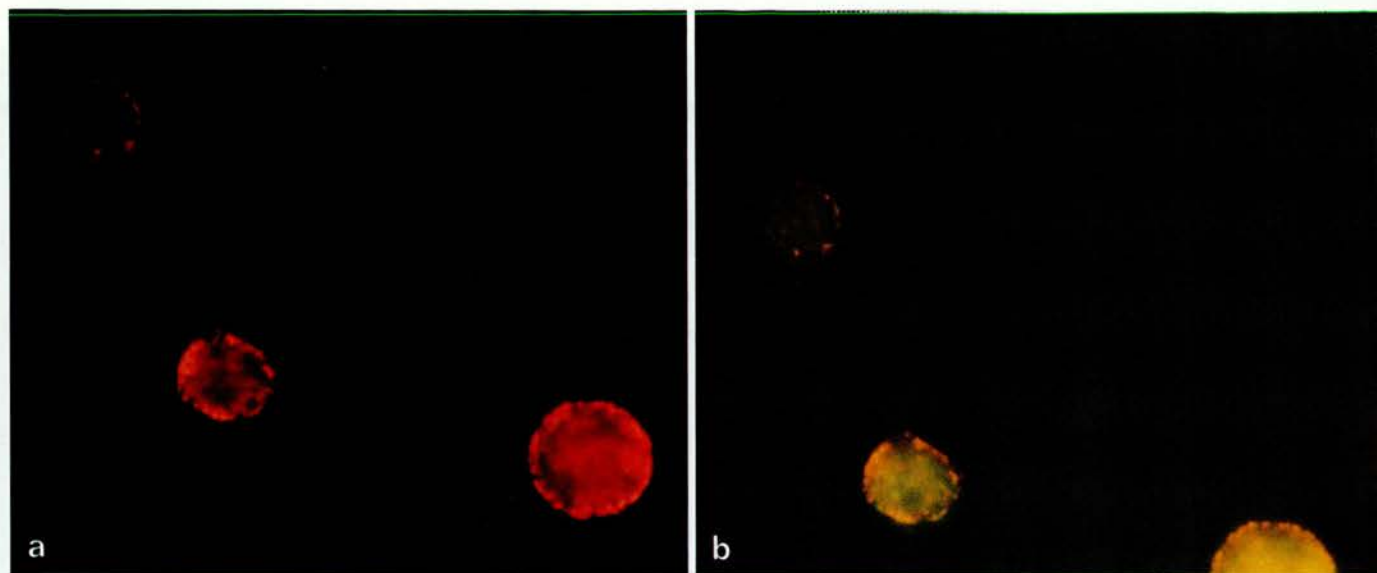


Fig. 1 a and b. Cell suspension (primary magnification $\times 400$) from BB/E/H rats cultured with r IFN- γ and stained by immunofluorescence using monoclonal antibodies. **a** Expression on all 3 cells in this field; **b** Double exposure photograph taken successively using normal green and red filters on a Leitz fluorescence microscope showing that the 3 class II positive cells contain insulin.

Islet cell immunofluorescence staining

The following monoclonal antibodies were generously provided by Dr. D. Mason, Sir William Dunn School of Pathology, Oxford: MRC OX-6, which recognises a non-polymorphic determinant on rat Ia, MHC Class II antigen; MRC OX-8, which recognises T cytotoxic lymphocytes and was used as a control for non-specific binding. Clone supernatants were used at 1:5 dilution in PBS (0.05 mol/l pH 7.6) containing 10% normal rat serum and L-lysine (20 mmol/l, Sigma). Islet cells (2×10^4) were incubated in the monoclonal antibody (100 μ l) at 0–4°C for 1 h. Following washing in PBS (0.05 mol/l, pH 7.6) containing L-lysine, (20 mmol/l) surface staining was revealed after a similar incubation with rhodaminated rabbit anti-mouse immunoglobulin (Dako Ltd., High Wycombe, Bucks, UK) previously adsorbed with rat liver acetone powder (Sigma). After washing, surface-stained cells were fixed in ice-cold 2% paraformaldehyde in 0.9% NaCl containing HEPES (20 mmol/l) and BSA, (1 mg/ml) pH 7.4 for 30 min. The cell suspensions were washed and put on to glass slides, air-dried and treated with 50% ethanol for 15 min at -20°C and washed again in PBS. Slide preparations were incubated for 40 min at 25°C with clone supernatants of monoclonal anti-insulin (2 FI), anti-glucagon (GLU 001) or anti-somatostatin (SOM 018) antibodies (kindly donated by Novo, Copenhagen, Denmark). Reaction was revealed using biotinylated horse anti-mouse immunoglobulins (Sera-Lab, Crawley Down, Sussex, UK) followed by fluoresceinated streptavidin (Cambridge Bioscience, Cambridge, UK). Slides were examined under a fluorescence microscope (Leitz, Luton, UK) equipped for double fluorochrome studies. Photographs were taken on Ektachrome ASA 400 film with automatic exposure.

Experimental protocol

Batches of pooled islets obtained from 4 animals on each occasion were isolated, cultured and stained from: 1) ICSA positive BB/E/H rats on 5 separate occasions ($n = 20$); 2) ICSA negative BB/E/H rats on 4 separate occasions ($n = 16$); 3) BB/E/L rats on 4 separate occasions ($n = 16$); and 4) normal Wistar rats on 3 separate occasions ($n = 12$).

Results and discussion

In agreement with other groups [4], induction of class II MHC antigen by r IFN- γ was never observed on any islet cells obtained from normal Wistar rats. Additionally, we found that r IFN- γ failed to induce expression of class II antigen on the surfaces of insulin containing B cells from BB/E/L animals. In contrast, islet B cells from BB/E/H animals, whether ICSA positive or negative, expressed class II antigen following culture with the same dose of r IFN- γ (Fig. 1). In agreement with our previous findings [1] class II expression was not induced on glucagon or somatostatin secreting islet cells from BB/E/H animals.

In our experiments 80% of the endocrine cells in any field were B cells, and on average 50% of these were induced to express class II antigen. Incubation of islet cells with the irrelevant monoclonal antibody MRC OX-8 (as a check for non-specific binding) produced no cell surface staining. OX-6 positive non-endocrine cells were seen only occasionally in any of the cell suspensions, which was not unexpected, since maintenance of isolated islets in free-floating culture for several days has been shown to act as a purification step for removal of non-endocrine cells [8].

Since expression of class II MHC antigen was not observed on any islet cells cultured in the absence of r IFN- γ , the expression of Ia antigen on B cells from BB/E/H animals is unlikely to be due to adsorption of shed Ia from passenger macrophages. These findings therefore suggest that the ability of r IFN- γ to induce class II expression may depend on genotype. Genetic factors could influence islet cell susceptibility to r IFN- γ .

by several possible mechanisms. Islets obtained from diabetes prone (BB/E/H) animals may have been damaged already; for example, by cytokines such as Tumour Necrosis Factor or Interleukin 1 produced by activated macrophages. The presence or absence of ICSA does not appear to be related to the sensitivity of B cells to γ IFN- γ . However, since circulating ICSA levels fluctuate in the pre-diabetic period it is likely that some animals categorised as ICSA negative had been ICSA positive at some time previously.

It has been suggested that aberrant expression of class II antigen on endocrine cells may be an initiating feature of autoimmune disease [2, 9]. However, our previous studies in the BB/E rat led us to postulate that expression of class II antigen on pancreatic B cells was a consequence rather than a cause of pancreatic mononuclear cell infiltration [1]. Since IFN- γ is produced only by T cells, our present findings add further weight to our concept of the sequence of events leading to the development of overt insulin-dependent diabetes.

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Pre-diabetes in the spontaneously diabetic BB/E rat: pancreatic infiltration and islet cell proliferation

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Abstract. A cohort of BB/E rats derived from litters with a high and low incidence of IDDM was studied prospectively to examine the relationship between circulating autoantibodies, islet insulin secretion, pancreatic infiltration, and islet cell replication during the pre-diabetic period. Although a higher incidence of islet cell surface (ICSA) and insulin autoantibodies (IAA) was detected in the diabetes-prone than in the low diabetic-incidence BB/E rats there was no correlation between the two antibodies in individual animals. Moreover, ICSA, but not IAA, were associated with loss of first phase islet insulin release. Between 75 and 105 days of age the number of diabetes-prone rats with ICSA and impaired islet insulin secretory function increased. Over the same period, there was a concomitant increase in the proportion of diabetes-prone animals with pancreatic infiltration, and increased islet endocrine cell proliferation. All these interrelated phenomena were observed in diabetes-prone BB/E rats at a time when the animals were normoglycaemic.

The spontaneously diabetic insulin-dependent BB rat displays a diabetic syndrome closely resembling that of human type I (insulin-dependent) diabetes mellitus (IDDM) in several important aspects Nakhoo et al. (1977). In particular, there is a genetic predisposition linked to the major histocompatibility complex RT1 (Colle et al. 1981), a long pre-diabetic period, and a mono-

nuclear cell pancreatic infiltrate associated with selective destruction of islet β -cells (insulinitis), which invariably precedes and accompanies the appearance of 'clinical' diabetes. The pathogenesis of the disease has been extensively investigated and there is evidence (reviewed by Yale & Marliss 1984) suggesting an involvement of cellular and humoral immunity in the destruction of β -cells. These findings together with the reported prevention of diabetes in this animal model by immunosuppression in the pre-diabetic period (Laupacis et al. 1983; Boitard et al. 1985) confirm its importance as a tool for defining precisely the sequence of events leading to the development of Type I diabetes.

Loss of pancreatic β -cell mass leading to overt IDDM results from a negative balance between islet cell proliferation and destruction. Attention has focussed on the latter process and there is no information about β -cell regeneration as spontaneous IDDM develops. We have therefore examined directly the relationship between circulating autoantibodies, islet morphology, and β -cell function/replication in the pre-diabetic period.

Materials and Methods

Animals

The animals used in this study were from the Edinburgh colony (subsequently designated BB/E), the nucleus of which was kindly donated in 1982 by Dr Pierre

This work was presented in part at the 20th Annual Meeting of the European Association for the Study of Diabetes, 12-15 September 1984, London, UK.

Table 1.

Parental phenotype	No. of litters	Normoglycaemic study rats (age in days)						Rats not included in study		Total
		30	45	60	75	90	105	Diabetic prior to study	Sacrificed age 120 days (status)	
Low diabetic-incidence										
ND × ND	4	4	4	4	4	4	2	0	7 12* (all ND)	41
Diabetes-prone									*retaining for breeding	
D × ND	10	8	8	8	8	9	13	21	11 (4 ND) (7 D)	86
D × D	3	4	4	4	4	3	1	2	1 (D)	23
Total	17	16	16	16	16 ^a	16 ^b	16 ^c	23	31	150

Excluded from study: ^a 3 rats; ^b 1 rat; ^c 3 rats (N × D mating) diagnosed diabetic on study day.
D: diabetic. ND: non diabetic.

Thibert from the colony maintained at the Animal Resources Division of Canada, Ottawa (designated BB). The BB/E colony consists of two sub-lines of animals created by selective outbreeding. The diabetes-prone line, predominantly maintained by crossing diabetic males with non-diabetic females, has a diabetic incidence of approximately 60% with a mean age at onset of diabetes of approximately 96 days. The low diabetes incidence subline has an incidence of diabetes of < 2% at 120 days of age. Details of the cohort of BB/E rats used in the study are shown in Table 1. Groups of 16 normoglycaemic rats were selected (as shown in Table 1) for study every 15 days between 30 and 105 days of age.

Protocol

At each time point, selected animals received an ip injection of tritiated thymidine (0.5 µCi/g body weight) one hour before being killed under light halothane anaesthesia. The pancreata (splenic portion) were then rapidly excised and plasma samples were taken for determination of plasma glucose (glucose analyser – Beckmann Instruments Inc, Fullerton, CA), insulin concentration, and autoantibodies to islet cell surface (ICSA), cytoplasm (ICA), and rat insulin (IAA). A portion of the excised pancreatic tissue was fixed in Bouin's solution for autoradiography and examination of mononuclear cell infiltration. The remainder was used to prepare isolated islets for insulin secretion studies in a multichannel perfusion system.

Autoantibodies

Plasma samples were applied undiluted to cryostat sections (4 µm) of normal Wistar rat pancreas for

detection of ICA using fluoresceinated goat anti-r: immunoglobulins (Nordic, Maidenhead, Berks, UK); the second layer. ICSA were estimated using a ¹²⁵I-protein A radioligand assay Dyrberg et al. (1982). Briefly, dispersed islet cell suspensions were prepared from β-cell-rich foetal rat islets and were incubated with plasma samples for 60 min at 4°C. The cells were washed and incubated for a further 30 min at 4°C with ¹²⁵I-protein A. After washing and centrifuging, the radioactivity in the cell pellet was determined in a gamma scintillation counter (Nuclear Enterprise, Edinburgh, UK). Cell-bound IgG was expressed as cpm/10⁵ islet cells. Intra- and inter-assay variation were 6% and 14%, respectively (N = 8) with non-specific binding (islet cells incubated in absence of plasma) contributing < 0.02% of total counts. Animals were regarded as being ICSA-positive when counts bound – 3 SD exceeded mean binding values (± 3 SD) for normal Wistar (518 ± 159 cpm/10⁵ cells; N = 32) and low diabetes incidence BB/E rats (486 ± 138 cpm/10⁵ cells; N = 132). IAA were measured by a modified direct immunospecific enzyme-linked immunosorbent assay (Elisa) (Dean et al. 1987), employing purified rat insulin (Novo Industri A/S, Copenhagen, Denmark) and horseradish peroxidase conjugated rabbit anti-rat immunoglobulins (Miles Laboratories, Stoke Poges, Slough, UK). Reference plasma from normal Wistar and diabetic BB/E rats were included in each assay as controls. Intra- and inter-assay variation were 5.6% (N = 5) and 15.2% (N = 3), respectively. Plasma samples were considered IAA-positive when OD values were two times the mean non-specific binding value and mean ± 3 SD binding for normal Wistar rats (OD = 0.014 ± 0.01; N = 12; aged 90–150 days).

Islet insulin secretion

The numbers of age-matched ICSA-positive and negative rats did not allow a full statistical evaluation of islet insulin release, but we considered it important to confirm that islets from the present study animals showed a pattern of insulin secretion similar to that observed in a previous study of diabetes-prone BB/E rats Bone et al. (1983).

Thus, islets were isolated from pooled pancreatic tissue of ICSA-positive or negative rats sacrificed at 75, 90 and 105 days of age. On each experimental day the islet insulin secretion was determined for up to 8 batches of 30 islets (4 channels each from the ICSA-positive and ICSA-negative groups) and the data combined to compare the pattern of insulin secretory response of ICSA-positive and ICSA-negative rats. The multichannel system and experimental protocol used for islet perfusion have previously been described in detail (Ashby & Shirling 1980). Briefly, the islets were equilibrated by a 40-min perfusion with basal media (2.7 mmol/l glucose) and then subjected to two consecutive stimulations with 16.7 mmol/l glucose applied 20 min apart. Samples of the perfusate were collected throughout and stored at -20°C until assayed for insulin. At the end of the second high glucose stimulation period, the islets were perfused for a further 20 min with basal media containing no BSA and recovered from the perfusion chambers. The islets were ultrasonically disrupted, and their protein content determined (Bio-Rad protein assay kit, Richmond, CA). Insulin secretion was expressed as $\mu\text{U IRI per } \mu\text{g islet protein}$ so as to correct for any possible differences in the size of islets isolated from ICSA-positive and negative animals.

autoradiography

Incorporation of tritiated thymidine into DNA during the S phase of the cell cycle has been widely used as an index of islet cell proliferation (Logothetopoulos 1972). autoradiography is considered to be the most sensitive method for detecting tritiated thymidine incorporated into islet DNA (Hellerstrom 1977).

Pancreatic paraffin sections (5 μm) of Bouin fixed tissue were mounted on gelatinised slides. The slides were dipped in Kodak NTB2 photographic emulsion and exposed for five weeks at 4°C . After developing for 5 min in Kodak D19 and fixing for 10 min in Kodak 24, the emulsion layer was allowed to dry overnight and the sections were then lightly counterstained with haematoxylin and eosin. The labelling index was determined by counting the number of radioactively labelled islet cell nuclei and expressing this value as a percentage of the total number of islet nuclei scored. Evaluation of sections containing several islets ensured that a minimum of 800–1000 nuclei were examined for each individual pancreatic tissue sample. Pancreatic sections were also examined for the presence of mononuclear

Table 2.

Plasma glucose and insulin concentrations in BB/E rats during the pre-diabetic period.

BB/E rats (age in days)	Plasma glucose (mmol/l)	Plasma insulin (mU/l)
30 (N = 16)	7.0 ± 0.2	4.9 ± 0.8
45 (N = 16)	8.2 ± 0.1	9.3 ± 1.3
60 (N = 16)	8.7 ± 0.1	21.7 ± 1.8
75 (N = 13)	8.0 ± 0.2	26.5 ± 7.4
90 (N = 15)	8.1 ± 0.2	27.2 ± 3.6
105 (N = 13)	8.2 ± 0.2	17.6 ± 2.1

Results expressed as mean values \pm SEM for the numbers of animals given in parentheses. *P* values indicate significant differences between mean values for the groups of animals shown (Student's unpaired *t*-test).

cell infiltration. All observations were performed blind on coded samples.

Samples of pancreas from a subsequent biopsy study (Bone et al. 1986) were examined to confirm that a possible proliferative activity of infiltrating cells was not contributing towards the islet cell labelling index recorded in those rats showing pancreatic infiltration. Cryostat sections of pancreas from 2 confirmed pre-diabetic BB/E rats (biopsied 10 and 13 days prior to onset of IDDM) were pre-stained with a monoclonal antibody to rat class II antigens and subjected to autoradiography as described above. The infiltrating mononuclear cells showing class II antigen expression did not possess radiolabelled nuclei whether located either at the periphery of islets or at a distance from islet tissue.

Results

Table 2 shows the plasma glucose and plasma insulin concentrations of the study animals, details of which are given in Table 1. Plasma glucose concentration increased significantly in the groups of animals from 30–45 days of age but thereafter remained constant at 8 mmol/l. There was a five-fold increase in the mean plasma insulin concentration between 30 and 75 days of age, the values remained steady at 90 days, and declined at 105 days of age. There were no significant differences in either plasma glucose or insulin values between age-matched low diabetic incidence and diabetes-prone rats.

Cytoplasmic ICA were not detected in any ani-

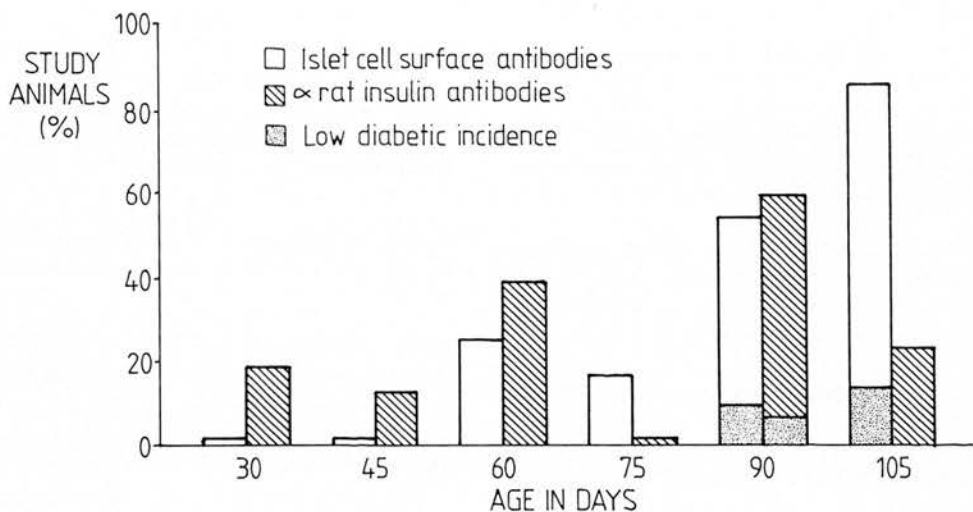


Fig. 1.

Percentage of BB/E rats with circulating islet cell surface and rat insulin antibodies in the different age group studied. The numbers of low diabetic-incidence and diabetes-prone animals per group are detailed in Table 1.

mal. The numbers of study animals with ICSA and IAA at the ages studied are shown in Fig. 1. ICSA were not detected in diabetes-prone animals prior to 60 days of age, but were demonstrated with increased frequency thereafter (60 days = 4/12; 75 days = 2/9; 90 days = 6/11; 105 days = 9/11 animals). IAA were found in diabetes-prone rats at 30 (3/12), 45 (2/12), 60 (6/12), 90 (8/11) and 105 (3/11) days of age. There was no correlation ($r = 0.104$) between IAA and ICSA in individual study animals.

Circulating ICSA and IAA were detected in only a small number of the low diabetic-incidence rats.

The insulin secretory response to glucose was compared between pooled batches of islets isolated from groups of ICSA-positive and ICSA-negative rats aged 75, 90 and 105 days. Findings from three experiments indicated a diminished total secretion of insulin in islets from ICSA-positive rats ($414 \pm 74 \mu\text{U IRI}/\mu\text{g islet protein}$) compared with islets from animals without ICSA ($577 \pm 72 \mu\text{U IRI}/\mu\text{g islet protein}$) with first phase release (98 ± 24 vs $151 \pm 16 \mu\text{U IRI}/\mu\text{g islet protein}$) being particularly affected.

Fig. 2 shows pancreatic autoradiographs from a low diabetic-incidence (A) and a diabetes-prone (B, C) rat both aged 75 days. The section from the low diabetic-incidence animal shows no mono-

nuclear cell infiltration and no labelled islet cell nuclei. Mild peripheral infiltration and many centrally located, radiolabelled islet cell nuclei are present in the pancreas of the diabetes-prone rat.

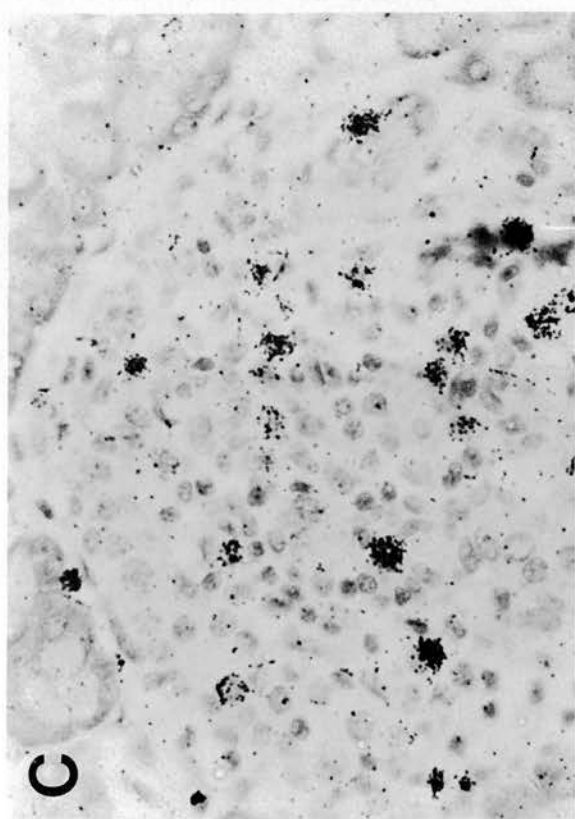
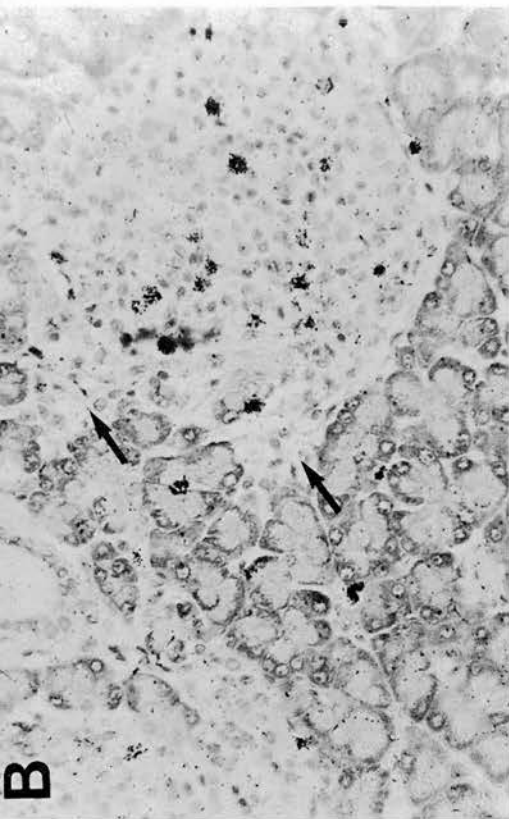
Fig. 3 shows labelling index values and the presence of pancreatic infiltration in all individual study animals. The labelling index was remarkably constant at or below 1% in the low diabetic incidence rats throughout the age range. The diabetes-prone rats showed a similar 1% labelling index up to 75 days of age, but at 90 and 105 days 4/11 and 6/10 rats, respectively, showed a labelling index of greater than 4%. These animals with a high labelling index also showed some degree of pancreatic infiltration. However, mononuclear cell infiltration was also observed in 7 diabetes-prone rats not showing an increase in labelling index values.

Discussion

In this study we have investigated the regulation of islet growth during development of IDDM in the spontaneously diabetic BB/E rat. We have therefore assessed in parallel, 1) metabolic status; 2) occurrence of circulating islet cell and insulin autoantibodies; 3) islet cell function (both insulin



B



C

Fig. 2.

Autoradiographs of paraffin sections of pancreas from normoglycaemic, low diabetic-incidence and diabetes-prone BB/E rats; prepared as described in Materials and Methods.

A. Normal islet showing no mononuclear cell infiltration and no labelled islet cell nuclei (low diabetic-incidence rat aged 75 days; haematoxylin and eosin $\times 300$).

B. Islet showing only mild, peripheral infiltration with no obvious accumulation of non-endocrine cells within the islet core (diabetes-prone rat aged 75 days; haematoxylin and eosin $\times 300$).

C. High power view of B showing numerous islet cell nuclei containing a mass of electron-dense grains ($\times 475$).

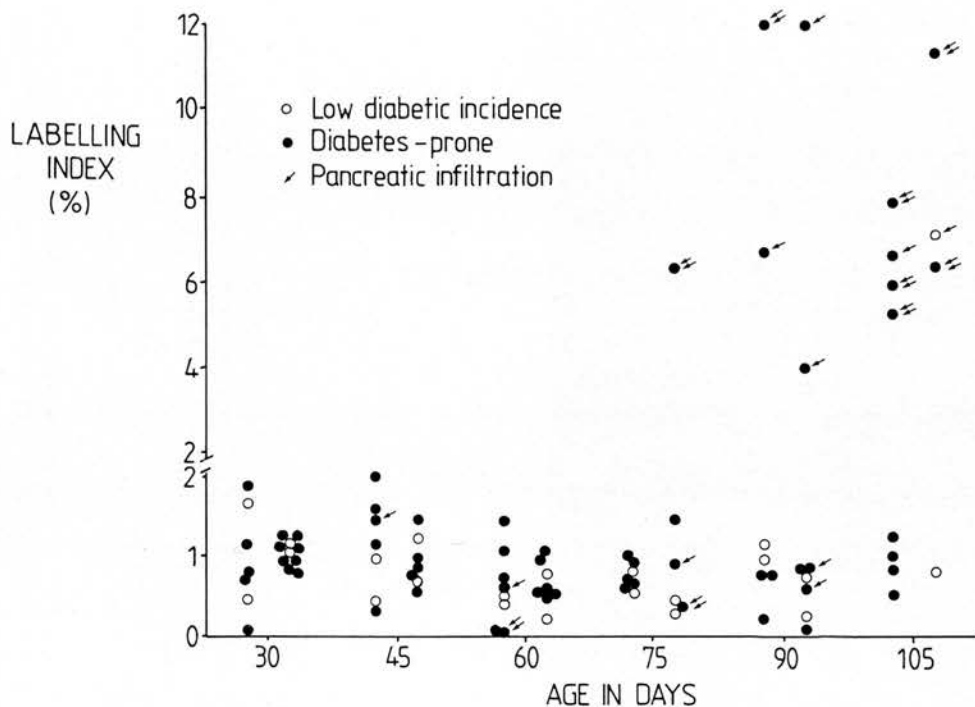


Fig. 3.

Islet cell proliferation values (labelling index) and pancreatic infiltration for each individual low diabetic-incidence and diabetes-prone rat studied at the ages shown. Data at each age point is arranged with values for male and female rats shown to the left and right hand side, respectively.

secretion and cell replication; and 4) pancreatic morphology in individual, potentially-diabetic BB/E rats prior to the development of 'clinical' diabetes. All findings being recorded at a time when the animals were normoglycaemic and had normal insulin levels.

In agreement with previous findings (Dyrberg et al. 1982, 1984), we were unable to detect ICA in any animal. ICSA and IAA were detected predominantly in diabetes-prone animals but there was no correlation between the presence of these two autoantibodies in individual rats. In addition, of the 19 animals showing pancreatic infiltration, 11 possessed circulating ICSA whilst only 5 had IAA. Our findings confirm the previously reported presence of ICSA in potentially diabetic BB rats (Dyrberg et al. 1982, 1984). On the other hand, our evidence would not appear to support the hypothesis that IAA are potentially useful markers for either autoimmune insulinitis or imminent development of diabetes in BB rats (Diaz et al. 1986).

The present islet insulin secretion studies, whilst not showing full statistically significant differences between ICSA-positive and negative rats, did indicate a perturbed pattern of biphasic insulin release in the ICSA-positive animals. A lower pancreatic insulin release has been reported in young potentially diabetic BB rats (Svenningsson et al. 1986) but the pattern of release was normal and was shown to occur as a result of a lower pancreatic insulin content. Such an explanation cannot account for the present findings, since results are expressed per unit islet protein and islets from ICSA-positive and negative rats have similar relative insulin content (Bone et al. 1983). The observed differences in the pattern of insulin release between these two studies could, however, be explained either by different experimental technique (isolated islets vs perfused pancreas) or by age difference of the study animals. Interestingly, a progressive decline in first phase insulin release during an IVGTT has recently been reported in pre-diabetic BB rats 25–50 days prior

to onset of overt IDDM (Reddy et al. 1986). These authors also noted a similar impairment of β -cell function in some BB rats which remained normoglycaemic. Taken altogether, these studies suggest that diabetes-prone BB rats with circulating ICSA and/or abnormal islet secretory function often go on to develop overt IDDM, but this is not always the case.

Whilst previous studies have investigated islet growth regulation in response to the hyperglycaemia of diabetes (for a review see Hellerstrom 1977), nothing is known about islet cell replication during development of IDDM. We have therefore investigated islet cell replication in groups of normoglycaemic but potentially diabetic BB/E rats. Low diabetes-incidence and young (< 75 days of age) diabetes-prone BB/E rats showed a rate of islet cell replication comparable to that of normal rats (Blum et al. 1963). However, as diabetes-prone rats approached the mean age for onset of IDDM (approximately 96 days), a number of animals showed a marked increase in islet cell labelling. All of these animals showed some areas of mononuclear cell infiltration within the pancreatic sections although it should be noted that both the periphery and overall integrity of their islets remained intact. Increased islet cell labelling was not always associated with mononuclear cell infiltration, since 7 diabetes-prone rats with a pancreatic infiltrate had a normal islet cell labelling index. This finding provides indirect evidence precluding a possible contributory effect of actively proliferating infiltrating cells towards an elevated labelling index value. Indeed, examination of cryostat sections from a subsequent biopsy study confirmed that infiltrating cells expressing class II antigens do not possess radio-labelled nuclei and were therefore unlikely to be responsible for the increased islet cell labelling observed in diabetes-prone rats.

From the present study it is not possible to determine whether increased islet cell replication may have a protective or preventative effect on the onset of overt IDDM in the BB rat. However, since pancreatic infiltration was observed in the absence of a raised islet labelling index, it is tempting to speculate that autoimmune attack of the pancreas may, in some animals, be able to trigger an increase in the rate of islet cell replication. Such an increase may help to explain why some BB rats showing impaired glucose tolerance and histological evidence of insulinitis are able to

maintain normoglycaemia (Reddy et al. 1986). It is equally possible, however, that animals remained non-diabetic because the autoimmune attack was less severe rather than because of a compensatory increase in islet growth.

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Insulin autoantibodies, islet cell surface antibodies and the development of spontaneous diabetes in the BB/Edinburgh rat

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SUMMARY

The presence of insulin autoantibodies (IAA) and islet cell surface antibodies (ICSA) was sought in two longitudinal studies, involving BB/Edinburgh rats of high (BB/E/H, $n=157$) and low (BB/E/L, $n=61$) susceptibility to diabetes development. Both studies were designed to correlate pancreatic morphology with cellular and humoral immunity. In Study I, groups of eight male and eight female non-diabetic rats of the BB/E/H line were killed at 15 day intervals from 30–105 days and plasma samples were obtained by cardiac puncture. In study II, 61 BB/E/H and 41 BB/E/L rats underwent pancreatic biopsy 1–3 times from 30 days of age until onset of diabetes or 150 days, plasma samples being taken from the tail vein at biopsy. Both studies revealed a higher prevalence for ICSA than IAA in BB/E rats. Whereas a highly significant association of ICSA with diabetes development was observed in study II ($\chi^2=8.30$, $P<0.005$), IAA were associated with diabetes development only weakly ($P<0.03$, Mann-Witney U -rank test). No correlation between the presence of ICSA and IAA in individual rats was observed and IAA were not significantly associated with BB/E/H in preference to BB/E/L rats, although positive IAA values were significantly elevated in the former compared with the latter ($P<0.01$). These observations support the concept that IAA form part of a background of heightened autoimmunity against which frank diabetes develops in some animals.

Keywords BB rats insulin autoantibodies islet cell surface antibodies spontaneous diabetes

INTRODUCTION

The spontaneously diabetic BB rat provides a good animal model for human Type I diabetes, a disease with autoimmune features (Nakhoda *et al.*, 1977). Animals characteristically develop glycosuria and hyperglycaemia between 60 and 150 days of age, the incidence of diabetes being equivalent in the male and female animals. Evidence for an immunological basis of the diabetes in this model (reviewed by Yale & Marliss, 1984) is provided by the observed insulinitis and selective β -cell destruction in the pancreas and the ability to prevent the disease by immunosuppressive treatments such as anti-lymphocytic serum, neo-natal thymectomy, anti I-E antibody administration or cyclosporine therapy.

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As in man, diabetes develops in the animals against a background of heightened autoreactivity, multiple autoantibodies being detectable during the prediabetic period. These include antibodies to islet cell surface (ICSA) and gastric parietal cells, thymocytotoxic antibodies and antibody to smooth muscle (Dyrberg *et al.*, 1982; Elder *et al.*, 1982). The precise relationship of these autoantibodies to the development of diabetes, however, is uncertain. Recently, insulin autoantibodies (IAA) have been detected in newly diagnosed untreated patients and in normoglycaemic potentially diabetic individuals (Palmer *et al.*, 1983; Arslanian *et al.*, 1985; Srikanta *et al.*, 1985). The predictive value of IAA for clinically overt diabetes is at present in dispute as published reports conflict (Karjalainen *et al.*, 1986; Dean *et al.*, 1986; Atkinson *et al.*, 1986).

One value of a model such as the BB rat is that it is possible to carry out longitudinal studies in which various parameters can be assessed for their predictive power. We report here the results of two such longitudinal studies in which plasma samples have been assayed for the presence of both IAA and ICSA and their significance for diabetes development analysed.

MATERIALS AND METHODS

The animals used in this study were from the Edinburgh colony (BB/E), the nucleus of which was kindly donated in 1982 by Dr P. Thibert, Animal Resources Division of Canada, Ottawa. This colony now has two components which have been created by selective breeding over the past 3 years; high susceptibility litters (BB/E/H) in which the average incidence of diabetes is 60–70%, with the mean age at onset 96 days, and low susceptibility litters (BB/E/L), in which the incidence of diabetes at the time of experimentation was below 10%.

Plasma samples

The plasma samples tested in this study were derived from rats used in two longitudinal studies designed to correlate pancreatic morphology with cellular and humoral immunity. Study I involved 96 BB/E/H animals with batches of eight male and eight female rats being killed at 15 day intervals from 30–105 days. In this group, samples were obtained by cardiac puncture at killing. For comparative values, plasma samples were obtained likewise from 20 BB/E/L animals (aged 90–120 days), 12 normal Wistar rats (90–150 days), and 32 diabetic BB/E/H rats (90–150 days) treated daily with insulin (Ultratard, Novo, Copenhagen).

Study II consisted of 102 animals (61 BB/E/H; 41 BB/E/L rats) which were biopsied 1–3 times between 30 and 150 days of age and also at onset of diabetes if symptoms developed. Plasma samples were obtained from the tail vein at the time of pancreatic biopsy.

Insulin antibodies

A micro-enzyme-linked immunosorbent assay (ELISA) as described for the measurement of human IAA (Dean *et al.*, 1986) was used with the following modifications:

Insulins. Microwells were coated using 100 ng purified rat, human or pig insulin (kindly donated by Novo).

Conjugate. Rabbit anti-rat immunoglobulin horse radish peroxidase (Miles Scientific, Slough, UK) was employed at 1:10³ dilution in 0.015 M PBS containing 0.1% Tween 20 (poly-oxyethylene sorbitan monolaurate, Sigma, Dorset, UK) and 10% heat-inactivated normal rabbit serum (NRS, Sera Laboratories, UK).

Test plasma and blocking for non-specific binding. Rat plasma were screened at 1:50 dilution in 0.015 M PBS containing 5% Tween 20 and 10% NRS. All microwells were blocked for non-specific binding by overnight incubation in PBS containing 20% NRS.

Substrate. Reactions with the substrate 3,3',5,5'-tetramethyl benzidine were terminated after 30 min incubation at room temperature. Samples were screened in triplicate in both antigen-coated and control wells. The latter were pre-treated with coating buffer alone (0.05 M Na carbonate-bicarbonate buffer, pH 9.6) and blocked for non-specific binding in the same manner as the antigen-coated wells. It was established that the presence of heparin or insulin in the physiological/diabetic range did not affect the assay.

Calculation of results

For each sample, the mean specific Optical Density at 450 nm (Δ OD) was calculated by subtraction of the mean non-specific reading in the corresponding control wells. Reference plasma from normal Wistar and diabetic BB/E rats were included in each assay as controls. (The intra- and inter-assay C.V. were 5.6% ($n=5$) and 15.2% ($n=3$) respectively, using rat insulin and a standard diabetic rat plasma.

The specific binding observed for plasma samples from normal Wistar rats ($n=12$, age 90–150 days) was Δ OD = 0.014 ± 0.018 , 0.071 ± 0.015 and 0.070 ± 0.013 (mean \pm s.d.) for rat, human and pig insulin respectively. Readings for other samples were considered positive when OD values were at least twice the corresponding mean non-specific binding value and greater than mean + 3 s.d. of the normal Wistar rat group for the relevant species of insulin.

Islet cell surface antibodies

ICSA were estimated using an ^{125}I -protein A radioligand assay essentially as described by Dyrberg *et al.* (1982). Dispersed islet cell suspensions were prepared from β -cell-rich fetal rat islets (Bone & Swenne, 1982) and 5×10^4 cells were incubated with plasma samples (10 μl ; final dilution 1:20) for 60 min at 4°C. The cells were then washed in PBS and incubated for a further 30 min at 4°C with ^{125}I -protein A. After washing and centrifuging, the radioactivity in the cell pellet was determined in a gamma Scintillation counter (Nuclear Enterprises, Edinburgh, UK). Cell-bound IgG was expressed as ct/min/ 10^5 cells. Intra and interassay variation was 6% and 14% respectively ($n=8$), with non-specific binding (islet cells incubated in the absence of plasma) contributing <0.02% of total counts.

Statistical analyses

The Mann–Witney *U*-rank test has been employed for the analysis of associations of IAA within the various groups studied, $P=0.05$ being chosen as the level of statistical significance.

RESULTS

The incidence of insulin autoantibodies in rats. Both studies showed the spontaneous occurrence of IAA in untreated diabetes prone BB/E rats. The results of study I are summarized in Table 1, from which it can be seen that a low incidence of IAA was observed from 30 days of age, with the peak incidence of activity occurring at 90 days, close to the mean age of onset of diabetes in the BB/E/H rat colony (96 days). IAA were also found in BB/E/L rats aged 90–120 days, but were never observed in outbred Wistar rats of comparable age. The presence of IAA in BB/E rats was confirmed in the serial biopsy study (study II). Only 4/39 (10%) and 1/31 (3%) of the high and low incidence lines respectively were positive for IAA before 60 days of age. Thereafter, 16/48 (33%) of biopsied BB/E/H rats were IAA positive, temporal variation in the presence of IAA occurring in eight of these animals; 3/29 (~10%) of the BB/E/L rats biopsied after 60 days of age were IAA positive, but Δ OD values were low in these three rats (3–4 s.d. > mean of Wistar rat values).

Table 1 also shows the species binding specificity of the insulin antibodies. Most of the rat IAA appeared to recognize shared epitopes on rat, human and pig insulin. Although human and pig insulin appeared more reactive than rat insulin in this assay system, among the 18 IAA positive BB/E/H rats (study I), six plasma samples bound preferentially to rat insulin, three to human and one to both pig and human insulins. As expected, a higher incidence of insulin antibodies was seen in diabetic animals maintained on injections of heterologous insulin.

Islet cell surface antibodies, insulin autoantibodies and diabetes development. The prevalence of ICSA with age in BB/E/H rats is shown in Table 1. Unlike insulin and other autoantibodies (such as parietal cell and smooth muscle antibodies) measured in this group (study I), ICSA were not observed before 60 days of age, but were found with increasing incidence rising to >75% at 105 days. In study I, there was no correlation ($r=0.104$) between ICSA and IAA in individual BB/E/H rats.

The distribution of ICSA and IAA in relation to diabetes development in study II is presented in Fig. 1. As in study I, no animal younger than 60 days old was positive for ICSA, so only data from rats aged 60–105 days have been included for analysis. Among this group 28 (58%) of the BB/E/H and four (14%) of the BB/E/L rats subsequently developed diabetes. In the high incidence line, 20/

Table 1. Incidence of antibodies to insulin and islet cell surface (ICSA) in BB/Edinburgh rats (study 1)

Age (days)	n	Insulin			ICSA (%positive)
		Rat (% positive per group)	Human	Pig	
BB/E/H rats					
30	16	13	13	6	0
45	16	0	0	0	0
60	16	13	13	38	25
75	16	31	25	38	25
90	16	38	63	56	56
105	16	19	31	31	75
BB/E/L rats					
90–120 days	20	35	45	45	NT
Insulin treated diabetic BB/E rats					
90–150 days	32	56	88	91	NT

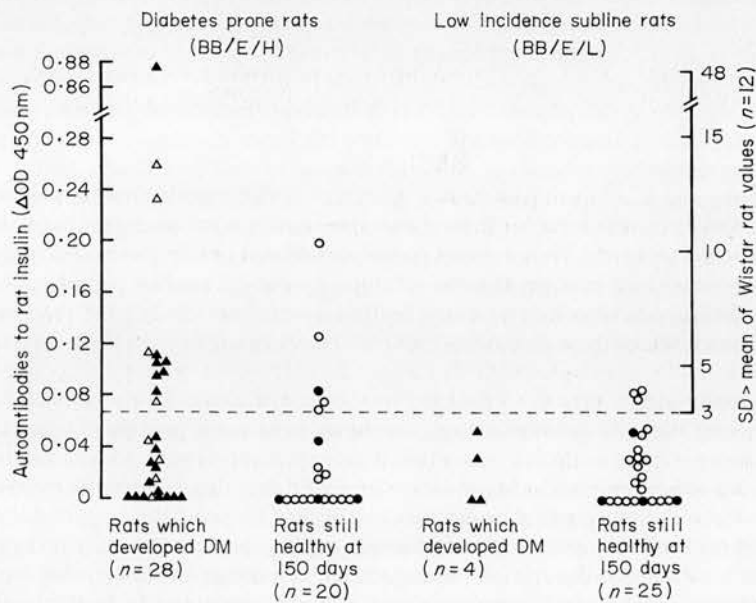


Fig. 1. Insulin autoantibodies (IAA) in relation to diabetes development. The presence of islet cell surface antibodies is indicated by solid symbols in high (BB/E/H) and low (BB/E/L) susceptibility rats (study II). The highest ΔOD values observed for IAA (rat insulin) in individual animals at any age between 60 and 150 days (or diabetes onset) is shown in relation to the number of s.d. above the mean of normal Wistar rat values ($n=12$), values > 3 s.d. (—) being regarded as IAA positive.

28 (71%) of the rats that became diabetic had ICSA, whereas only seven (35%) of these rats were also IAA positive. By contrast, in the group of 20 BB/E/H rats which did not develop diabetes, four had ICSA, a further four were IAA positive and one animal alone was positive for both antibodies. In the BB/E/L rats, all four animals which developed diabetes were ICSA positive/IAA negative, but among the 25 animals of this group that remained healthy at 150 days, four had ICSA and a further three rats had IAA (albeit of low value).

A strong association of ICSA with diabetes development was thus observed in the biopsy study ($\chi^2=8.30$, $P<0.005$). Insulin autoantibodies were less significantly associated with diabetes development ($P<0.03$), using Mann–Witney *U*-rank test analysis of values from all study II rats which developed diabetes ($n=32$) versus those remaining healthy ($n=45$), and this low level of significance was confirmed ($P<0.05$) if BB/E/H rats alone were considered in the analysis.

Similar analysis of the data from BB/E/H rats (Fig. 1) revealed no significant association ($P=0.4$) of IAA with ICSA positive ($n=25$) versus ICSA negative animals ($n=23$), confirming the earlier observation in study I. Likewise, no significant association ($P=0.15$) was found for the presence of IAA in BB/E/H rats ($n=48$) compared with BB/E/L rats ($n=29$), presumably due to the low incidence of IAA in these animals. A further analysis, based solely on all positive IAA values recorded in the biopsy study, however, shows that higher ΔOD values for IAA are significantly associated ($P<0.01$) with the BB/E/H observations ($n=47$) compared with those for the BB/E/L rats ($n=19$).

DISCUSSION

Tolerance to self antigens can be broken following administration of an antigen crossreactive with self (Weigle, 1965), thus thyroglobulin or insulin autoantibodies are induced in genetically susceptible mice or humans following injection of rat thyroglobulin or pig insulin. These induced autoantibodies arise as the result of the provision of a new carrier determinant on the immunogen to which the recipient is not tolerant. This situation generates carrier specific T helper cells, which can help autoreactive B cells, recognizing shared determinants on the cross-reacting antigen. The occurrence of insulin autoantibodies in established diabetic BB/E rats could therefore be viewed as a breach of self-tolerance by this process, since these animals are maintained on heterologous insulin. However, the presence of autoantibodies in untreated BB/E rats and their absence in age-matched Wistar rats requires further explanation. Although tolerant to their own insulin, healthy rats would not be expected to be tolerant to pro-insulin, which is normally sequestered in the β -cell. In the prediabetic period of the BB/E rat, we have shown that insulinitis (and attendant β -cell destruction) occurs from 60 days onwards. Thus, it is not inconceivable that insulin precursors (pre-proinsulin and proinsulin) come into contact with the immune system during this period, and this could generate an autoantibody response, which by definition would be wholly cross-reactive with pro-insulin. The occurrence of these autoantibodies may be restricted to animals of a certain genetic background.

Insulin autoantibodies have been detected in the sera of non-diabetic individuals. In man, complement fixing islet cell antibodies are thought to have some predictive value for insulin-dependent diabetes. Wilkin *et al.* (1985) have found no association of human insulin autoantibodies with the presence of islet cell antibodies and have suggested that their presence is more compatible with individual genetic susceptibility rather than established disease. However, Dean *et al.* (1986) have examined the class distribution of these autoantibodies and have found that the presence of both IgG insulin autoantibodies and complement fixing islet cell antibodies confers increased risk for future diabetes development than the presence of either marker alone. In the BB/E rat, although we observed a preferential association of antibodies to rat insulin with the group of animals which subsequently became diabetic, the finding was of low significance and there was no correlation between the presence of these antibodies and islet cell surface antibodies, which proved a much better indicator for diabetes development. However, subclass analysis of the spontaneous rat insulin autoantibodies will have to be performed before any definitive statement can be made.

Autoantibodies can arise not only through antigenic cross reactivity but also via perturbations of the idiotypic network. For example, studies by Wasserman and his colleagues (1983) have shown in animals that administration of BISQ (an acetyl choline agonist) results in the production of autoantibodies to the acetyl choline receptor. Additionally Cohen and his coworkers (1984) have shown that administration of insulin to genetically susceptible mice leads to the production not only of insulin autoantibodies, but also to antibodies to the insulin receptor, presumably via perturbation of the idiotypic network. We have previously already discussed the ways in which

autoantibodies to the insulin receptor or to insulin could arise following viral infection (Cooke, Lydyard & Roitt, 1985) and a recent report (Bodansky *et al.*, 1986) has demonstrated a high incidence of IgM class insulin autoantibodies following such common viral infections as mumps, measles, chickenpox and rubella. Whether these arise as a consequence of viral mediated destruction of the β -cell with concomitant release of insulin precursors leading to insulin autoantibodies as described above or via perturbation of the idiotypic network as hypothesized (Cooke *et al.*, 1984), remains to be elucidated. If the autoantibodies prove to be wholly cross reactive with insulin precursors, β -cell attack by a virus would appear to be the initiating event. A viral aetiology of the disease in the BB rat cannot be excluded. Examination of serial plasma samples for autoantibodies to insulin and the insulin receptor should at least allow us to decide which autoantibody arises first and which develops as a consequence of the idiotypic network.

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Immunological responses of the BB rat colony in Edinburgh

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SUMMARY

Several immunological responses of the spontaneously diabetic BB rat colony in Edinburgh designated (BB/E) have been studied. The proliferative responses to Con A and LPS, ability to make IL-2 and to show NK activity have been studied using diabetic and non-diabetic BB/E rats and normal Wistar rats. Our data suggest that the diabetic animals in the BB/E colony do not have marked deficiencies in any of these parameters. Lymphopenia and depressed T-cell responses do not appear to be a prerequisite for the development of diabetes in the BB/E colony.

INTRODUCTION

BB rats are a partially inbred line of rats that have a high incidence of development of insulin-dependent diabetes mellitus (Review by Yale & Marliss, 1984). This disease has an autoimmune involvement and makes the BB rat a good animal model for human Type 1 (insulin-dependent) diabetes mellitus. Several workers have demonstrated immunological deficiencies in the diabetic BB rat (Bellgrau *et al.*, 1982; Maclaren *et al.*, 1983; Prud'Homme *et al.*, 1984; Guttman *et al.*, 1983), and Gille, Guttman & Seemayer (1981) have shown lymphopenia and diabetes to be genetically linked in the BB rat. However, this has not been a uniform finding, and since we are attempting to dissect those factors that determine whether an animal progresses towards the development of diabetes, it was important for us to ascertain the immunological status of the BB colony in Edinburgh. We describe in this paper our comparative studies of some immunological parameters in the diabetic BB/E rat.

MATERIALS AND METHODS

Animals
The Edinburgh colony of BB rats (BB/E) was derived from a nucleus which was kindly donated in 1982 by Dr P. Thibert, Animal Resources Division of Canada, Ottawa. The BB/E colony has been selectively inbred to generate two lines: the high incidence, diabetes-prone animals (incidence of diabetes is 60–100% with the mean age of onset at 96 days) and the non-diabetic subline (now in its eighth generation with a very low incidence of diabetes, <4%). Wistar rats used in these experiments were obtained from Olac 1976 Ltd, Bicester, Oxon.

The animals used in these experiments were established diabetic rats, non-diabetic subline rats and Wistar rats that were age-matched.

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Proliferation assays

Spleen cells from individual rats were washed three times in BSS and cultured at 2×10^5 cells per well in 96-well, flat-bottomed microtitre plates (Sterilin, Teddington, Middlesex) in RPMI-1640 (Flow Laboratories, Irvine, Ayrshire) supplemented with 5% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin G and 100 µg/ml streptomycin (final volume 200 µl). The mitogens used were concanavalin A (Con A) from ICN Biomedicals (High Wycombe, Bucks) used at doses indicated in the experiment, and lipopolysaccharide (LPS) from Difco Laboratories (West Molesey, Surrey) used at a final concentration of 20 µg/ml. After 48 hr at 37°C in a humidified atmosphere of 5% CO₂ in air, the cultures were pulsed with 0.5 µCi ¹²⁵I-deoxyuridine (Amersham International, Amersham, Bucks) in 50 µl RPMI. Eighteen hours later, the cultures were harvested onto glass fibre discs using a Titertek cell harvester (Flow Laboratories) and incorporated radiolabel assessed by gamma counting.

IL-2 release assay

Spleen cells were cultured as above with 2 µg/ml Con A. After 24 hr 100 µl of supernatant were removed from each well, and IL-2 was measured by its ability to maintain the growth of CTL-L cells (Kendal Smith, Dartmouth Medical School, Dartmouth, New Hampshire) at 2×10^4 cells per well (assessed in a 24-hr proliferation assay).

NK assay

The NK-sensitive cell line YAC-1 was grown in RPMI-1640 with 10% (v/v) FCS and supplements as above. ⁵¹Cr-labelled YAC-1 were plated out at 10^4 cells per well into flat-bottomed 96-well microtitre plates with fresh spleen cells (effector cell ratios of 12.5 : 1, 25 : 1, 50 : 1 and 100 : 1) in a final volume of 200 µl in RPMI plus 10% FCS and supplements as above. After 4 hr of incubation at 37°C, 100 µl of supernatant were removed

from each well and counted on a gamma counter. The percentage specific lysis was calculated by:

$$\% \text{ specific lysis} = \frac{\text{c.p.m. experimental release} - \text{c.p.m. spontaneous release}}{\text{c.p.m. maximum release} - \text{spontaneous release}} \times 100.$$

RESULTS

Response to Con A

Responses of BB/E (diabetic and non-diabetic) and age-matched Wistar rat spleen cells are shown in Fig. 1. There was considerable variation in the responses of spleen cells from diabetic animals to Con A (Fig. 1b) but these responses were significantly lower than those found with cells from Wistar rats (Fig. 1a). However, this lowered response to Con A did not correlate with the diabetic status of the animals since spleen cells from the non-diabetic subline gave markedly poorer responses (Fig. 1c) than those from either Wistar or diabetic animals.

Ability of Con A-stimulated spleen cells to make IL-2

From Table 1 it can be seen that spleen cells from diabetic animals produce normal levels of IL-2 following stimulation with Con A. Both Wistar and diabetic rat spleen cells produced more IL-2 than those from the non-diabetic subline. It is interesting to note that one animal in the diabetic group appeared to be spontaneously releasing IL-2, which probably reflects the presence of activated T cells *in vivo*.

Response to LPS

The ability of rat spleen cells to respond to LPS was assessed and the data are presented in Table 1. Spleen cells from all these rat strains responded significantly but poorly to LPS, no difference being detected between those spleen cells derived from diabetic or normal age-matched control animals.

NK activity

Previous work has suggested that the numbers of NK cells may be elevated in diabetic rats (Marliss, Gross & Yale, 1985;

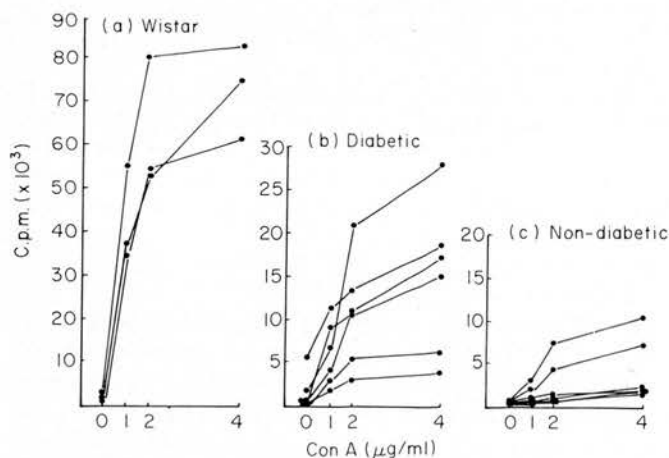


Figure 1. Each line represents an individual animal. All groups are significantly different from each other ($P < 0.01$).

Table 1. IL-2 release and LPS responses

Rat number	Response measured			
	IL-2 release*		LPS (20 μg/ml)	
	-Con A	+Con A	-LPS	+L
Wistar				
1	368 ± 103	10,377 ± 760	2758 ± 226	4840 ± 100
2	1511 ± 96	7826 ± 138	1415 ± 314	4180 ± 100
3	240 ± 48	14,652 ± 1020	557 ± 40	1354 ± 100
Non-diabetic (BB/E)				
1	296 ± 29	4422 ± 89	791 ± 213	1429 ± 100
2	354 ± 79	5051 ± 269	418 ± 19	1357 ± 100
3	405 ± 55	4514 ± 1421	636 ± 201	1451 ± 100
4	640 ± 157	2460 ± 162	581 ± 102	1742 ± 100
5	190 ± 18	4528 ± 581	431 ± 83	1252 ± 100
6	227 ± 46	2450 ± 181	300 ± 18	1080 ± 100
Diabetic (BB/E)				
1	573 ± 120	7266 ± 320	455 ± 44	1368 ± 100
2	516 ± 122	9269 ± 3046	5889 ± 42	7261 ± 100
3	686 ± 89	7113 ± 297	1876 ± 356	7397 ± 100
4	2067 ± 158	5884 ± 371	741 ± 36	2086 ± 100
5	13,578 ± 662	14,812 ± 251	527 ± 29	1461 ± 100
6	2188 ± 961	6873 ± 105	476 ± 56	1453 ± 100

* Twenty-four hour supernatants were used at 50% in the assay.

† Twenty-four hour assay using the CTL-L line at 2×10^4 /well. V and diabetic were not significantly different, but both were different from the non-diabetic group ($P < 0.001$).

‡ Seventy-two hour assay with 2×10^5 spleen cells/well. Wistar and diabetic were not significantly different but both were different from non-diabetic group ($P < 0.001$).

Rabinovitch, Mackay & Boulton, 1985; Woda *et al.*, 1986. T NK activity in spleen cells from the BB/E (diabetic and non-diabetic) and Wistar rats was therefore measured using YAC cells as targets. From Fig. 2 and Table 2 it can be seen that no significant difference in NK activity at any effector to target ratio was demonstrated using spleen cells from diabetic and non-diabetic BB/E and normal rats.

DISCUSSION

Studies on Con A responses in BB rats (Jackson *et al.*, 1981; Yale & Marliss, 1984) have produced conflicting data. The findings that responses to Con A were low in rats with insulinitis but normal in rats without insulinitis (Rossini *et al.*, 1979) could provide one explanation for the discrepancies. Bellgrau (1983) found normal responses in BB diabetic and non-diabetic animals to Con A, but allogeneic responses were not found in mixed lymphocyte cultures. Prud'Homme *et al.* (1984) attributed this defect in the response of BB spleen cells to Con A to suppressor macrophages. Our results suggest that BB/E diabetic rats can make a significant response to Con A and do not manifest any defect related to diabetic status.

In our studies, normal levels of IL-2 could be demonstrated in supernatants from diabetic spleen cells following Con

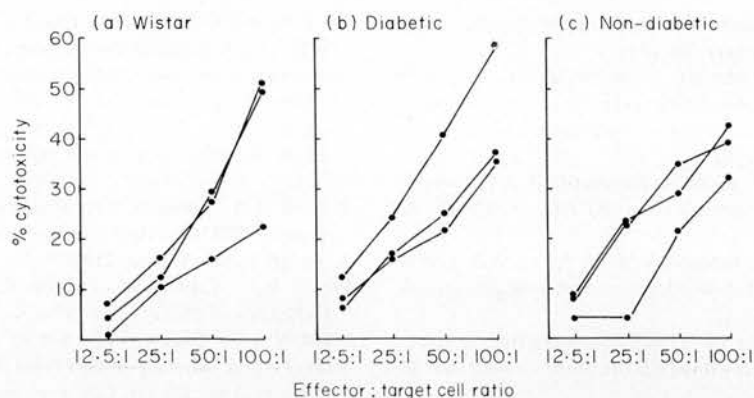


Figure 2. Each line represents an individual animal assayed at various effector: target ratios. There is no significant difference between any of the groups.

Table 2. NK assay

Rat number	% cytotoxicity
Wistar	
1	49.5 ± 2.7
2	51.6 ± 2.6
3	22.7 ± 1.7
Non-diabetic	
1	32.2 ± 3.3
2	39.5 ± 0.9
3	42.5 ± 5.2
Diabetic	
1	38.6 ± 3.7
2	36.7 ± 4.2
3	35.7 ± 1.8
4	18.1 ± 4.5
5	57.9 ± 1.8
6	58.9 ± 1.4
7	36.4 ± 9.6
8	37.9 ± 3.5

Four hour ^{51}Cr -release assay, with an effector: target ratio of 100:1.

Groups are not significantly different

the majority of animals used in this study and only observed one animal with endogenously activated T cells.

The cell-surface marker studies of Marliss, Grose & Yale (1985) showed an increased population of cells (possibly NK cells) in diabetic animals, which was not present in non-diabetes-prone BB rats. Recently Woda *et al.* (1986) showed an increased number of OX8⁺, OX19⁻ (NK) cells in diabetes-prone and acute diabetic rats. The results from our study show comparable levels of NK activity in all three types of rats, although slight variations between individual spleens were observed. We conclude that NK cell activity does not appear to be deficient or elevated in the spleens of diabetic BB/E rats.

Our data suggest that the diabetic animals in the BB/E colony, unlike those in other BB rat colonies, do not manifest marked deficiencies in their ability to respond to T-cell or B-cell mitogens, to make IL-2 or to generate NK activity. Lymphopenia and depressed T-cell responses do not therefore appear to be a prerequisite for the development of diabetes in the BB/E colony. Yale & Marliss (1984) have suggested that the immunological disorder is more severe in BB rats when compared to humans, since man does not have lymphopenia or functional T-cell defects. Thus, the BB/E colony may resemble human Type 1 diabetes mellitus more closely than other colonies previously described.

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mulation. This confirms the data of Maclaren *et al.* (1983) and der & Maclaren (1983) but conflicts with those of Prud'homme *et al.* (1984) who found a defect in IL-2 release. While saying for IL-2 release following Con A stimulation, spontaneous IL-2 release following simple culture of spleen cells was obtained. One of the diabetic animals spontaneously produced large amounts of IL-2, suggesting the presence *in vivo* of preactivated T cells. Francfort *et al.* (1985) have shown activated T lymphocytes in pre-diabetic BB (diabetes-prone) rats, the presence of which peaks before onset of diabetes and declines with onset. These authors conclude that elevated Ia-positive T lymphocytes may be a marker for susceptibility to diabetes. Since all the diabetes-prone rats used were established diabetics, we may have missed the activated T-cell response in

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III.8

Aberrant expression of class II MHC molecules on pancreatic B-cells during development of insulin-dependent diabetes: cause or consequence?

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The major histocompatibility complex (MHC) antigens are cell surface glycoproteins, associated with the regulation of immune responses. These molecules are divided into two main groups: class I antigens, located on most nucleated cells and responsible for restriction of T-cytotoxic lymphocyte responses and class II molecules, required for antigen presentation and normally present only on the surface of antigen-presenting cells such as macrophages, activated T-cells and B-lymphocytes¹. The observation that class II molecules were expressed on follicular cells in autoimmune thyroid disease², and that this expression could be induced on normal thyroid cells³ by the lectin phytohaemagglutinin, led to the hypothesis that aberrant expression of class II antigens may allow the presentation of specific auto-antigens to potentially autoreactive T-helper lymphocytes, thereby leading to autoimmune disease⁴. Such aberrant expression of MHC class II antigens on pancreatic B-cells has been described in human type I diabetes⁵. In this report of a single case with recent onset of type I diabetes, examination of fresh frozen autopsy pancreas revealed two major abnormalities of MHC expression in some islets: firstly expression of class II MHC products specifically confined to pancreatic B-cells and secondly marked hyperexpression of class I MHC molecules on all types of endocrine cells. Similar abnormalities of MHC expression were found in the pancreases of 35 patients who had died with type I diabetes and showed classical histopathology, namely mononuclear cell infiltration of islets (insulinitis).⁶ Aberrant expression of class

II MHC molecules was observed on the surface of pancreatic B-cells in some islets and hyperexpression of class I MHC molecules was seen in most insulin-containing islets. Whereas, all insulin-containing islets affected by insulinitis hyperexpressed class I MHC antigens, some were seen with insulinitis but no class II expression on B-cells, and others showed class II MHC expression on B-cells in the absence of insulinitis. While recognizing the limitations imposed by cross-sectional data collected at a time when 80–90% of B-cells were already destroyed, Foulis *et al.*⁶ proposed a possible sequence of preceding events occurring within individual islets which culminated in specific destruction of B-cells, namely hyperexpression of MHC class I molecules followed either directly by insulinitis with no aberrant expression of class II MHC antigens by pancreatic B-cells or by aberrant expression of class II molecules on B-cells followed by insulinitis. Finally the inflammatory process leads to destruction of B-cells leaving an islet deficient in insulin-containing cells and no longer hyperexpressing class I MHC molecules.

Direct study of the sequential changes occurring in the pancreas in the prediabetic period is, however, only possible using an animal model such as the spontaneously diabetic, insulin-dependent BB rat⁷ which has a long prediabetic period (>3 months) during which time selective destruction of islet B-cells occurs⁸. The degree of insulinitis varies between animals but in all cases is observed well in advance of the onset of overt hyperglycaemia. Using this animal we performed a longitudinal study designed to examine the relationship between markers of autoim-

munity, islet morphology and B-cell function in the prediabetic period^{9,10}.

Expression of MHC class II molecules in the BB/E rat pancreas

The nucleus of the Edinburgh colony of BB rats (BB/E) was kindly donated in 1982 by Dr Pierre Thibert from the colony maintained at the Animal Resources Division of Canada, Ottawa. The BB/E colony consists of two sublines of animals created by selective breeding. The diabetes-prone BB/E subline (DP) has an incidence of diabetes of 56–65% and a mean age at onset of ~96 days. The diabetes-resistant BB/E subline (DR) has an incidence of diabetes of zero in the last two generations. A cohort of 96 BB/E rats was studied prospectively with eight male and eight female normoglycaemic rats being selected for study every 15 days between 30 and 105 days of age. At each time point the selected animals were killed and the pancreas removed. The pancreatic tissue was examined for the presence of a mononuclear cell infiltrate and processed for autoradiography for investigation of islet cell replication. Serum samples were also taken and stored at -20°C for determination of glucose and the presence of autoantibodies. Using monoclonal antibodies (Mab) specific for rat lymphocyte subsets and MHC class II molecules, the frozen sections of pancreas were extensively screened for the distribution of subpopulations of lymphocytes infiltrating the pancreas and also for the presence of Ia molecules on endocrine cells.

The first change observed in the pancreas of normoglycaemic DP animals was increased expression of MHC class II molecules on vascular endothelial cells and this preceded any mononuclear cell infiltration. Next, T helper cells (identified by co-expression of W3/25 and OX19 antigens) infiltrated the pancreas around blood vessels and these infiltrating T-cells were expressing class II molecules indicating their activation. In older DP rats the pancreatic infiltrate also contained T-cytotoxic/suppressor cells and B-lymphocytes. Finally in pancreases showing severe insulinitis, expression of class II molecules was occasionally detected on B-cells which still contained insulin in partially destroyed islets. Expression of class II molecules was never observed on glucagon or somatostatin containing cells⁹. The incidence of islet cell surface (ICSA) and insulin autoantibodies (IAA) was higher in DP than in DR rats, but there was no correlation between the two antibodies in individual animals. The proportion of DP

rats with circulating ICSA increased as the mean age of onset of diabetes approached. Over the same time period there was a concomitant increase in the proportion of DP rats with insulinitis and an associated increase in islet cell proliferation¹⁰. Our overall conclusion from these studies was that expression of MHC class II molecules on pancreatic B-cells did not precede lymphocytic infiltration and was therefore likely to be secondary rather than a primary initiating phenomenon.

In-vitro induction of MHC class II molecules

That class II MHC expression on insulin-containing islet cells was a consequence rather than a cause of islet infiltration was supported by subsequent in-vitro studies in which we investigated the role of cytokines in the induction of MHC class II molecules on islets isolated from BB/E rats. We found that islets isolated by collagenase digestion from 90-day-old DP rats, when cultured with 100 U/ml of rat recombinant interferon- γ (rIFN- γ) for >4 days, expressed MHC class II molecules on ~50% of the insulin-containing B-cells¹¹. In contrast, induction of expression of class II molecules by rIFN- γ was never observed on: (1) glucagon or somatostatin cells in islets from any animals; (2) B-cells from either normal Wistar or BB/E DR rats, or (3) B-cells from young (30-day-old) DP rats. This suggested the possibility that islets isolated from older DP rats might have been rendered susceptible to the induction of class II molecules by IFN- γ because of previous cytokine-mediated damage *in vivo*.

Some support for this concept was provided by the studies of Pujol-Borrell *et al.*¹², showing that IFN- γ in combination with tumour necrosis factor (TNF) or lymphotoxin could induce expression of MHC class II molecules on endocrine cells in monolayer cultures of human pancreas. However, since this cytokine combination also induced class II molecules on glucagon and somatostatin containing cells it is difficult to reconcile these findings with in-vivo observations in both human⁵ and BB rat⁹. Moreover, we have been unable to induce expression of MHC class II molecules on islets isolated from either normal Wistar or BB/E DR rats using TNF (both recombinant human and rat), as well as Interleukin 1 (IL-1) with or without rIFN- γ (unpublished). Although synergism between IFN- γ and other cytokines may occur¹³, there are studies suggesting that IFN- γ is sufficient by itself to induce class II molecules on isolated

murine¹⁴, and human¹⁵ islets. It is possible, however, that these effects may result from synergism of IFN- γ with cytokine products released from macrophages present in the cultures. Whereas, Campbell *et al.*^{16,17} and Pujol-Borrell *et al.*¹⁸ reported enhanced expression of MHC class I but not class II molecules on isolated mouse¹⁶ and human^{17,18} islets when cultured with IFN- γ alone, these authors used a much lower concentration of IFN- γ and a shorter culture period than that used in studies performed by Wright^{14,15}.

These disparate results suggest that the use of in-vitro studies to examine the possible role of cytokines in the destruction of pancreatic B-cells has limitations and caution must be exercised in extrapolations to the in-vivo situation. Variation in the source, species and concentration of cytokine employed and the conditions and duration of culture and cytokine exposure undoubtedly affect the results obtained and probably account for apparent discrepancies in the results reported from different centres.

Studies on the pathogenesis of diabetes in the BB/E rats using serial pancreatic biopsies

The longitudinal cohort study⁹ discussed above had several inherent limitations: (1) it was not known which animals with insulinitis would have gone on to develop IDD subsequently; (2) it was not possible to assess the time scale of the lymphocytic infiltration/B-cell destructive processes with any degree of accuracy; (3) the Mabs used to characterize the lymphocytic infiltrate were unable to distinguish between T-cell subsets and certain populations of macrophages. In an attempt to remedy these deficiencies we have employed serial pancreatic biopsy as described by Logothetopoulos *et al.*¹⁹. This allows sequential study of individual animals over prolonged periods. Groups of DP rats were biopsied between 40 and 100 days of age with each individual animal being biopsied twice at a 10-day interval. Rats developing diabetes were biopsied for a third time at onset of diabetes. The biopsied pancreatic tissue was immediately snap frozen and cryostat sections prepared for immunohistochemical examination. Sections were screened with Mab for both class I and class II MHC molecules and the subpopulations of cells comprising the mononuclear cell infiltrates were also examined. In addition to Mab identifying rat T- and B-lymphocytes, Mab directed against distinct antigenic determinants (ED1 and ED2) on rat macrophages²⁰ were also employed.

Macrophages in pathogenesis

A resident population of tissue macrophages (ED2 positive) was observed in all biopsy sections. These cells were observed around the perimeter of the pancreatic lobules and were also widely dispersed throughout the connective and acinar tissues. The distribution and numbers of ED2-positive macrophages did not vary between biopsies in individual animals and did not change as the mean age at onset of diabetes approached. The Mab ED1 revealed a separate population of pancreatic macrophages which were usually located either periductally or around islets and which in normal Wistar, BB/E DR and young (40–50 days of age) DP rats were much less numerous than ED2-positive cells. Although there was some variation in time scale in individual DP animals, between 14 and 21 days before onset of overt diabetes marked recruitment and accumulation of ED1-positive cells occurred in the pancreas at periductal, perivascular and peri-islet locations and this was followed by infiltration of islets by these cells before there was any apparent involvement of other immune effector cells. T-lymphocytes (OX19 positive) including T-helper cells, NK-cells and B-lymphocytes, were recruited into the areas of insulinitis, ~10 days before the onset of diabetes. At this time there was marked hyperexpression of MHC class I molecules on the surface of all islets cells. Expression of MHC class II molecules was, however, restricted to infiltrating immunocytes with no expression of MHC class II on pancreatic B-cells. Pancreatic biopsies obtained at onset of overt diabetes revealed almost total destruction of pancreatic B-cells with very few insulin-containing cells remaining. Double fluorochrome labelling revealed that only very occasionally were MHC class II antigens expressed in association with insulin-containing cells.

It is possible that our earlier observation of class II positive insulin-containing cells in end-stage islets⁹ may represent the phagocytic uptake of fragments of damaged insulin-containing cells by cells which are themselves expressing class II antigens²¹. These results support an earlier report describing an initial phase of single cell insulinitis, which on electron microscopy appeared to consist mainly of macrophages²². It is interesting that a virtually identical pattern of distribution of ED1 and ED2 macrophages was recently described in the central nervous system of rats with early experimental allergic encephalomyelitis²³. The suggestion that mac-

rophages may have an important role in IDD pathogenesis in the BB rat is supported also by the observation that diabetes can be prevented in this animal by administration of silica²⁴ which is known to block macrophage function.

Conclusive remarks

While the precise mechanism of the death of B-cells is still uncertain, the sequence of pancreatic events leading up to their mass destruction can now be described in the BB/E rat. The first phenomenon observed is hyperexpression of MHC class I molecules on islet cells. The cause of this is unknown but it has been suggested that chronic viral infection (which is known to be a common stimulus for the release of interferons from epithelial cells and has been shown to enhance expression of class I MHC molecules by pancreatic endocrine cells *in vitro*¹⁸) could be responsible²⁵. Subsequent infiltration of these islets by ED1-positive macrophages may release cytokines which could be directly cytotoxic to islet B-cells²⁶, and/or via local release of IL-1 and TNF activate the endothelial cells of pan-

creatic venules and ducts to bind increased numbers of lymphocytes²⁷. Infiltrating lymphocytes would then be attracted to islets already invaded by ED1 macrophages by a chemotactic gradient of IL-1²⁸. Within infiltrated islets presentation of B-cell specific antigens by activated ED1 macrophages to MHC class II restricted T-helper cells may then serve to initiate further immune mechanisms leading to the specific destruction of islet insulin producing B-cells. Our observations do not support the proposal that aberrant expression of MHC class II molecules on pancreatic B-cells has a primary initiating role in the process which ultimately leads to their destruction.

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III.9

Development of BB rat diabetes is delayed or prevented by infections or applications of immunogens

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The spontaneously diabetic BB rat has become an accepted model for insulin-dependent diabetes (IDD). Several lines of evidence suggest that immunological mechanisms are central to the initiation of a diabetic state resembling human IDD. Therefore, many studies were undertaken to prevent the development of diabetes in BB rats by modulating their immune system, e.g. by neonatal thymectomy¹, total lymphoid irradiation², treatment with anti-lymphocyte sera³, cyclosporine A⁴⁻⁶ and/or glucocorticoids⁵ and by transfusion of whole blood⁷, white blood cells⁸, or T-lymphocyte subsets⁹ from diabetes-resistant rats. The immune system is also influenced by factors from the natural environment, e.g. by microbes, toxins, or chemicals with stimulatory effects on the immune system. Some of them might act on the immune system in a way similar to that effective in artificial modulation. In man, diabetes concordance of 50% only in identical twins¹⁰ suggests that such natural factors are of importance. Observations in our BB rat colony have also supported this assumption. Two latent mycoplasma infections reduced the incidence of diabetes from 70.6 to 14.9% and from 77.3 to 38.6%, respectively¹¹. The increase of diabetes incidence from 38.6 to 92.8% in the first generation after their transfer into a germ-free environment using hysterectomy confirmed the stimulatory action of mycoplasma as 'natural modulators' of the immune system. These facts prompted us to simulate the effect of exogenous immune stimuli by application of two weak immunogens—insulin and tuberculin—on the development of IDD in BB rats. Both immunogens were chosen because these BB rats are low- or non-responders to insulin, and tuberculin is a purified hapten-like protein.

Animals and methods

From the 11th inbred generation of our BB/OK rat colony, obtained from Dr Thibert's colony in Canada in 1983, 33 males and 59 females were used. These animals were the progeny of first and second litters of the same parents which descended from the same grandparents and served both as control and experimental animals as shown in Table 1. They were kept under standardized hygienic conditions, and were caged in groups of 4-6 animals in the same room as detailed before¹². Pelleted food (Rezeptur 13, Zentralinstitut fuer Ernährung, Rehbruecke, GDR) and water were provided *ad libitum*.

Crystallized bovine insulin (100 µg) dissolved in 0.1 ml saline or 0.1 ml tuberculin (PPD vet. 'Dessau', Institut fuer Impfstoffe, Dessau, GDR) were applied i.p. with 0.1 ml complete Freund's adjuvant (CFA) at 5 weeks of age. One week later, the experimental animals were given the same amount of immunogens without CFA twice per week until the 20th week of life. The control animals were treated only with 0.1 ml CFA at 5 weeks of age.

From the 3rd until the 32nd week of life the following parameters were monitored: body weight and plasma glucose weekly, polymorphonuclear leucocytes (PMNL) and lymphocytes at 4, 6 and 8 weeks and then monthly, glucose tolerance at 10, 15, 20, 25 and 30 weeks, percentage of infiltrated pancreatic islets (biopsy) at 20 and 31 weeks, host versus graft reaction (HVGR) at 20 and 32 weeks, and pancreatic insulin content at 32 weeks. The rats were considered diabetic if their plasma glucose concentration was >20 mmol/l.

Blood was taken by puncture of tail vein between 06.00 and 08.00 hours from non-fasting animals. Plasma glucose was measured on a

Distinct Macrophage Subpopulations in Pancreas of Prediabetic BB/E Rats

Possible Role for Macrophages in Pathogenesis of IDDM

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Use of monoclonal antibodies directed against rat macrophages and serial pancreatic biopsy in the prediabetic period have enabled us to document the involvement of macrophages in the pancreatic events leading to onset of diabetes in the spontaneously diabetic BB/E rat. A few weeks before onset of disease, there is marked recruitment and accumulation of ED1⁺ macrophages at periductal and perivascular locations adjacent to noninfiltrated islets. These recruited cells, distinct from the resident ED2⁺ tissue macrophages, then infiltrate the islets. Infiltration of the pancreas by ED1⁺ macrophages is therefore a very early event in the prediabetic period and suggests a possible role for macrophages in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) in this animal model. *Diabetes* 37:1301-304, 1988

The BB rat develops a diabetic syndrome closely resembling human insulin-dependent diabetes mellitus (IDDM) (1). In a prospective longitudinal cohort study in which batches of prediabetic BB/Edinburgh (BB/E) rats were killed at intervals in the prediabetic period, we have previously reported the presence of OX19⁺ (T-) and W3/25⁺ (helper T-) lymphocytes in the early stages of pancreatic infiltration (2). However, because W3/25 also recognizes certain macrophage populations (3), it is important to use a different marker to allow discrimination between T- lymphocytes and macrophages. Recently, several monoclonal antibodies (MoAbs) identifying distinct rat macrophage subpopulations have been described (4). We have used these along with serial pancreatic biopsy in in-

dividual animals to investigate a possible role for macrophages in the development of diabetes in BB/E rats.

MATERIALS AND METHODS

The animals used in this study were from the Edinburgh colony of BB rats, the nucleus of which was kindly donated in 1982 by P. Thibert (Animal Resources Division of Canada, Ottawa). The BB/E colony consists of two sublines of animals created by selective breeding. The diabetes-prone main line has a 60-70% incidence of diabetes, with the mean age of onset ~96 days. The diabetes-resistant subline has an incidence of diabetes of <1% at 120 days. Animals (20 male, 20 female) were selected at random from age-matched diabetes-prone litters. All animals were monitored daily in relation to body weight and glycosuria. Animals developing overt diabetes were maintained on a single daily subcutaneous injection of Ultratard insulin (Novo, Copenhagen). Animals from the diabetes-resistant subline and normal Wistar rats were used as nondiabetic and non-BB control rats, respectively.

Protocol. Pancreatic biopsy was performed essentially as described by Logothetopoulos et al.(5). Eight groups ($n=5$) of diabetes-prone animals were biopsied twice (with a 10-day interval) between 30 and 110 days of age. Animals developing diabetes received a third biopsy at onset of disease. Biopsies were also performed on diabetes-resistant animals ($n=12$) and on normal Wistar rats ($n=6$) between 100 and 120 days of age. Biopsies (50-75 mg of tissue) were immediately snap frozen in isopentane at -70°C for subsequent immunohistochemical examination. One hundred twenty serial cryostat sections ($4\text{ }\mu\text{m}$) were cut from each biopsy. Scan slides with 8 sections/slide, each section taken at a different level through $80\text{ }\mu\text{m}$ of tissue, were used as an initial screen to detect areas of pancreatic infiltration with class II MHC-positive cells. Detailed analysis of areas with infiltration via the antisera listed below were performed by referring back to subsequent serial sections coincident with the areas represented by the scan slides.

Antisera. The following MoAbs were generously provided as clone supernatants (D. Mason, Sir William Dunn School

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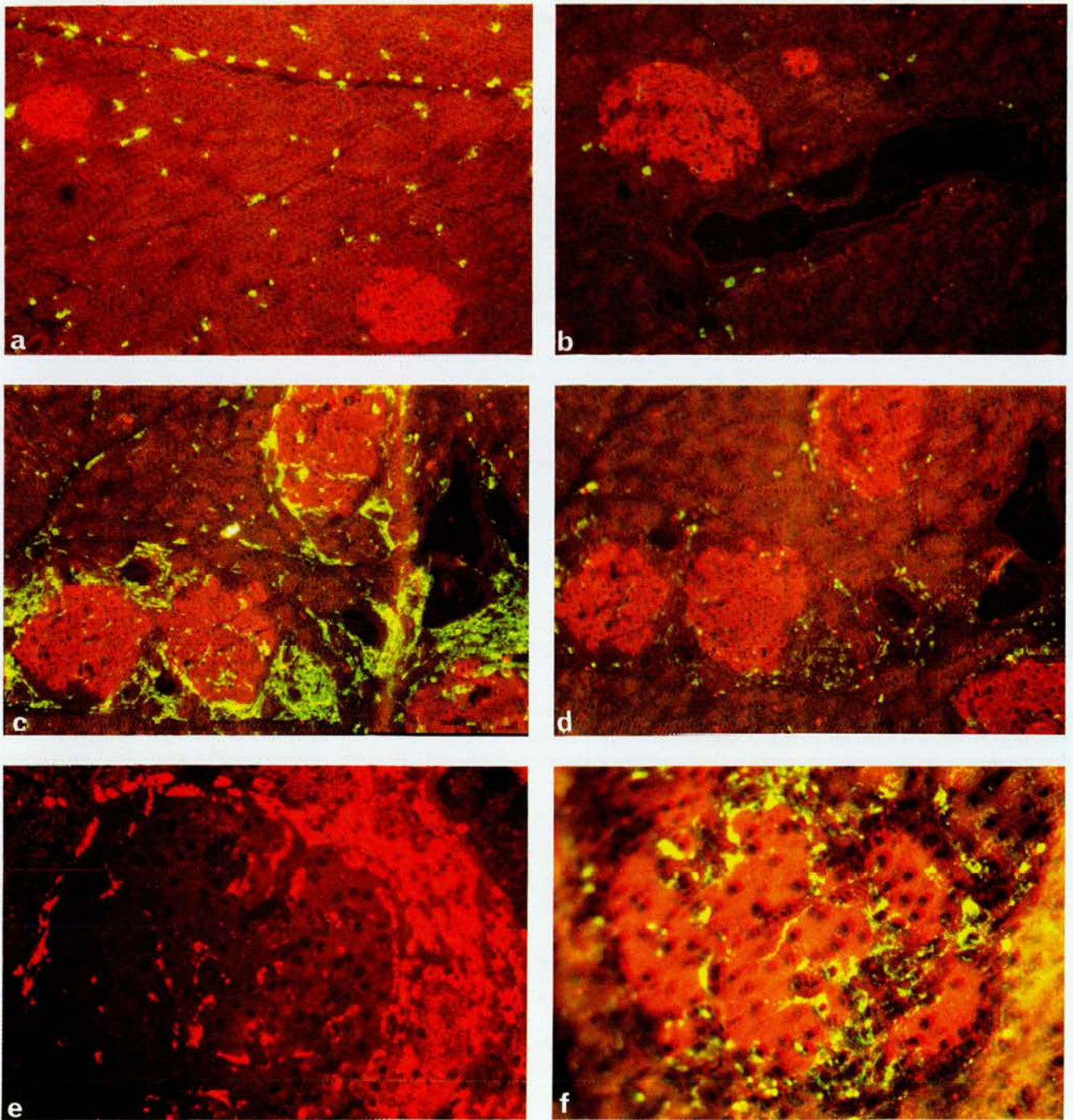


FIG. 1. Cryostat sections of pancreatic biopsies stained by indirect immunofluorescence with antibodies as described in MATERIALS AND METHODS. **A:** double exposure of ED2 (green) and insulin (red) staining on normal Wistar rat pancreas (aged 100 days). ED2⁺ cells are found prominent within septa of pancreatic lobules. $\times 125$. **B:** double exposure of ED1 (green) and insulin (red) in normal Wistar rat pancreas (aged 100 days). Occasional ED1⁺ cells observed periductally. Elsewhere in sections, few cells stain with ED1. $\times 125$. **C–E:** serial sections from biopsy of diabetes-prone animal (aged 70 days). Biopsy was taken 20 days before onset of diabetes. **C:** double exposure for cells expressing MHC class II molecules (green) and insulin (red). Four islets within same pancreatic lobule show early peri-islet infiltration; cells were recruited from vascular sites supplying the lobule. $\times 125$. **D:** serial section of **C** stained for ED1 (green) and insulin (red) shows many OX6⁺ cells to be ED1⁺ macrophages. $\times 125$. **E:** ED1 staining (red) at higher magnification ($\times 300$) of an islet shown in **D**. ED1⁺ cells accumulate at perivascular site, with mild peri-islet infiltration. **F:** double exposure of ED1⁺ cells (green) infiltrating insulin-containing islet (red). Biopsy taken from same animal 10 days later from sections shown in **C–E**, $\times 300$.

of Pathology, Oxford, UK): MRC-OX6 (rat Ia or MHC class II antigen); MRC-OX8 [cytotoxic/suppressor T-lymphocytes and natural killer (NK) cells]; MRC-OX19 (pan-T-lympho-

cytes); MRC-OX33 (rat leukocyte-common antigen, present only on B-lymphocytes); W3/25 (helper T-lymphocytes and macrophages). The MoAbs ED1 and ED2 directed against

distinct antigenic determinants on rat macrophages were obtained as ascites fluid (Serotec, Oxford, UK; 4). Novo supplied the polyclonal antiserum M1183, raised in guinea pig against insulin-containing cells. A rabbit anti-glucagon antiserum was obtained from Immunodiagnostics (Washington, UK).

Immunofluorescence staining. Cryostat sections (4 μ m) of biopsy samples were air dried, acetone fixed for 10 min, and screened with the antisera. Clone supernatants were used at 1:2 to 1:10 dilution in phosphate-buffered saline (PBS, pH 7.6) containing 10% normal rat serum. Ascitic fluids and polyclonal antisera were applied at 1:50 to 1:100 dilution in PBS containing 10% normal rat serum for ascites or 10% nonimmune serum from the host species in which the antisera were raised for the polyclonal antisera. Sections were incubated with the antisera for 60 min at 25°C. Reactions of MoAb binding were revealed with species-specific biotinylated sheep F(ab')₂ anti-mouse IgG (Amersham, Aylesbury, UK), followed by fluoresceinated or Texas red-conjugated streptavidin (Amersham). Endocrine cell markers were revealed with the corresponding fluoresceinated or rhodaminated antibodies to guinea pig or rabbit immunoglobulins (Cambridge Bio Science, Cambridge, UK). A 10-min washing period with 3 changes of PBS followed each application. After mounting, sections were examined under a Leitz Orthoplan microscope fitted with a fluorescence vertical illuminator and filter blocks for revealing fluorescein and rhodamine/Texas red fluorochromes. Photographs were taken on Ektachrome ASA 400 film with automatic exposure.

RESULTS AND DISCUSSION

Each biopsy can only reflect a part of the total pancreas at a single time point. However, by using scan slides to screen whole islets within many pancreatic lobules, we can describe changes in pancreatic morphology leading to insulinitis within individual and multiple islets.

The normal tissue distribution of cells identified by MoAbs ED1 and ED2 was assessed by use of pancreatic sections from normal Wistar rats and animals from the diabetes-resistant subline. ED2 revealed a resident population of tissue macrophages present in all biopsies from all groups of animals. These cells were prominent lining the septa between pancreatic lobules and were widely dispersed throughout the connective and acinar tissues but were not present within islets (Fig. 1A). Fluctuations in the degree of dispersion and number of ED2⁺ cells found between biopsies in individual animals were minimal and may have been a consequence of the biopsy. Biopsies from diabetes-prone animals at all ages showed a similar tissue distribution. There was no correlation between distribution of ED2⁺ cells and onset of diabetes in individual animals. Indeed, ED2⁺ cells were not found in any islets showing insulinitis.

In contrast, ED1 revealed a separate population of pancreatic macrophages distinct from ED2⁺ cells. In the normal control pancreases and biopsies from diabetes-prone animals at an age when insulinitis has never been observed (40 days), ED1⁺ cells were much less numerous than ED2⁺ cells. Occasionally, a few ED1⁺ cells were observed periductally or rarely at peri-islet sites (Fig. 1B).

Approximately 14–21 days before onset of diabetes, the first sign of mononuclear cell infiltration was observed in diabetes-prone animals at periductal, perivascular, and peri-

islet sites in some lobules (Fig. 1C). Further analysis with serial sections revealed that this infiltrate consisted mainly of ED1⁺ cells (Fig. 1, D and E). Initially this was focal, with most islets in one pancreatic lobule showing some degree of peri-islet infiltration (Fig. 1D), whereas islets in adjacent lobules could appear unaffected. This initial accumulation of ED1⁺ cells at perivascular locations was not a nonspecific consequence of the biopsy, because it was never observed in sequential biopsies from normal Wistar or diabetes-resistant BB rats. The recruited ED1⁺ cells then proceeded to infiltrate islets within affected lobules (Fig. 1F). In all cases, most initially infiltrating cells appeared to be ED1⁺, with substantial numbers of T-lymphocytes (OX19⁺), including helper T-lymphocytes (OX19⁺, W3/25⁺), NK cells (OX8⁺, OX19⁻), and B-lymphocytes (OX33⁺) seen only subsequently at ~10–14 days before the onset of diabetes. Progressive spreading of this insulinitis process from lobule to lobule will eventually result in overt diabetes.

These results support the observations of Kolb et al. (6), who have described "single-cell insulinitis" early in the pre-diabetic period, which on electron microscopy appeared to consist mainly of macrophages. Other ultrastructural studies have described macrophages containing ingested β -cell debris within islets of diabetic BB rats (7). In addition, the prominent appearance of ED1⁺ macrophages in the perivascular infiltrates associated with experimental allergic encephalomyelitis, another autoimmune disease (8), agrees with our observations.

It has been reported that the administration of silica, which blocks macrophage function, can prevent the onset of diabetes in BB rats (9). Our findings, together with the evidence cited, would suggest a possible role for macrophages in the pathogenesis of IDDM in the spontaneously diabetic BB/E rat. The precise nature of this role is still unclear.

Interestingly, a recent article has postulated that a population of intravascular monocytes may induce venular leakage and hence allow enhanced diapedesis of lymphoid cells from the circulation into the pancreas of susceptible BB rats (10). We speculate that this population of intravascular monocytes may correspond to ED1⁺ cells, because ED1 but not ED2 stains most peripheral blood monocytes (4), and ED1⁺ cells have been seen trapped in the vascular endothelium of Wistar rats (11). Having infiltrated the islets, ED1⁺ cells may release cytokines, e.g., interleukin 1 (IL-1) and tumor necrosis factor, which may be cytotoxic to islet β -cells (12,13) and/or may, via these mediators, activate the endothelial cells of pancreatic venules and ducts to bind increased numbers of lymphocytes (14). The infiltrating lymphocytes could then be attracted to islets already infiltrated by macrophages by a chemotactic gradient of IL-1 (15). Within the infiltrated islets, presentation of β -cell-specific antigens by ED1⁺ OX6⁺ macrophages to MHC class II-restricted helper T-lymphocytes may serve to initiate further immune mechanisms leading to the specific destruction of islet β -cells.

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